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EXPERIMENTAL PATHOLOGY

THE QUESTION OF SEROLOGICAL RACES OF *V. CHOLÉRÆ* AND THE RELATION OF SOME OTHER VIBRIOS TO THIS SPECIES.

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At one time it was almost universally believed that epidemic cholera was caused by a number of races of vibrios which could be separated from one another by small cultural and morphological differences; all, however, were included under the name of *V. choleræ*.

It was early recognised that certain serum tests—Pfeiffer's reaction, and agglutination tests—were the most certain methods of ascertaining whether or no the vibrio in question was capable of causing true epidemic cholera. However, before these tests were adjudged the necessary criterion of classing a vibrio as a true member of the species *V. choleræ*, a number of organisms had been isolated from various sources, such as well-water, *e. g. V. nasik*, or from the fæces of human beings not suffering from cholera—such as the various El Tor vibrios—which were culturally and morphologically very similar to the true *V. choleræ*.

These organisms were later investigated by serum tests, and somewhat confusing results were obtained by different workers.

More recently, from sporadic outbreaks of a cholera-like disease, usually recognisable from true epidemic cholera by the restricted area of the epidemic, milder symptoms and lower case mortality, vibrios have been isolated which in morphology, some cultural characters, and in their pathogenicity for the usual laboratory animals, resemble *V. metchnikovi* almost as closely as *V. choleræ*. Serologically, these organisms have been described as quite distinct from the true *V. choleræ* and have been separated into a number of serological races. This disease has been denominated paracholera and the organisms as *V. paracholeræ* A, B, etc., according to the serological grouping.

Under these circumstances, and in view of the work which has been carried out by Andrewes and Inman on the *B. dysenteriæ* (Flexner) group of organisms, it was decided to ascertain if a number of strains of *V. choleræ* showed any serological differences, and also to reinvestigate the serological characters of certain of the vibrios concerning which a variety of opinions have been expressed.

Nine strains of *V. choleræ* were obtained, eight having been collected at various times and from various sources by the Pathological Department of the Royal Army Medical College. Of these, one strain at least was obtained from Galicia by J. Freeman in 1915; three strains were originally obtained from the Pasteur Institute at Paris; the exact source of the remaining four was not known. The ninth strain was one which the Italian Government employed in the manufacture of the anti-cholera vaccine used by the Italian army during the late war.

From this last strain a vaccine was prepared and a series of doses of from 200 to 500 million vibrios was given intravenously to a rabbit. When preliminary tests showed that the serum had an agglutinating titre of about 800 to 1000, the animal was bled from the ear-vein, and the serum thus obtained stored in hermetically sealed small test-tubes, each containing about 3 c.c.

The first experiment was made to ascertain if, using this serum, emulsions from nine strains of *V. choleræ* were agglutinated to approximately the same titre. The emulsions were made by planting each strain into peptone water, incubating for from eighteen to twenty hours at 37° C. and then diluting the cultures thus obtained to a similar and convenient opacity with normal saline solution, sufficient formalin being added to make the concentration of the final emulsion 1 in 1000.

The agglutination tests were carried out in Dreyer's standardised tubes, 400 c.mm. of the serum dilution being mixed with 600 c.mm. of the formolised emulsion. The tubes were then placed in a water-bath at 50° to 55° C. Readings were made after the tubes had been at least two hours in the bath, and these were verified by a second reading made after the tubes had stood overnight at room temperature.

The results are shown in the following table:

TABLE I.

Strain.	Dilutions of serum, 1 in—								
	25	50	100	200	400	800	1600	3200	Control.
R.A.M.C. 2 . .	+	+	+	+	+	S.+	S.—	tr.—	—
R.A.M.C. 4 . .	+	+	+	+	+	S.	tr.	—	—
R.A.M.C. 5 . .	+	+	+	+	+	S.	tr.+	—	—
R.A.M.C. 6 . .	+	+	+	+	+	S.+	S.	tr.—	—
Galicia J.F. 7 .	+	+	+	+	+	S.	tr.	—	—
Pasteur Inst. 8 .	+	+	+	+	+	S.+	S.—	—	—
Pasteur Inst. M. 9 .	+	+	+	+	+	S.+	S.	—	—
Pasteur Inst. P. 10	+	+	+	+	+	S.+	S.	—	—
Italian 11 . . (homologous str.)	+	+	+	+	+	S.	tr.	—	—

The sign + indicates complete agglutination; — absence of agglutination; S. standard agglutination; tr. trace of agglutination.

Examination of these results shows that all the strains were agglutinated by very similar dilutions of the serum prepared by means of a vaccine made from the "Italian" strain.

Similar results were obtained when sera prepared by inoculating rabbits with vaccines made from the strains R.A.M.C. 2 and Galicia J.F. 7 were employed. The same technique was used. In Table II the results obtained with the latter serum are shown.

Strain Pasteur Inst. P. 10 was not tested.

TABLE II.

Strain.	Dilutions of serum. 1 in—								
	25	50	100	200	400	800	1600	3200	Control.
R.A.M.C. 2 . .	+	+	+	+	+	+	+	S.	—
R.A.M.C. 4 . .	+	+	+	+	+	+	S. +	tr.	—
R.A.M.C. 5 . .	+	+	+	+	+	+	S.	—	—
R.A.M.C. 6 . .	+	+	+	+	+	+	S.	—	—
Galicia J.F. 7 . (homologous str.)	+	+	+	+	+	+	S.	—	—
Pasteur Inst. 8 .	+	+	+	+	+	+	S.	—	—
Pasteur Inst. M. 9 .	+	+	+	+	+	+	S.	—	—
Italian 11 . .	+	+	+	+	+	+	+	S.	—

Although these results were so uniform it was thought necessary to confirm them by a series of tests in which the serum was tested for agglutinating power after it had been absorbed by thick emulsions of the various strains.

These tests were carried out as follows:

Agar slopes were planted with strain R.A.M.C. 2, Galicia J.F. 7, Pasteur Inst. M. 9, Pasteur Inst. P. 10 and Italian 11. After incubation overnight the resulting growths were washed off with 1 or 2 c.c. of salt solution to each agar slope. The opacity of these thick emulsions was then compared by making a series of dilutions in tubes of standardised bore; sufficient salt solution was then added, where necessary, to bring them all to, approximately, the same opacity.

Each emulsion was then divided into two portions, one of which was kept undiluted and the other was diluted 1 in 5 with salt solution. One c.c. of these undiluted emulsions and those diluted 1 in 5 respectively were then mixed with 1 c.c. of the serum, prepared by means of strain "Italian" 11, diluted 1 in 5. After being kept at room temperature for two hours the tubes containing the emulsions and serum were placed in the ice-chest overnight. As a control 2 c.c. of serum, diluted 1 in 5, was mixed with 2 c.c. of salt solution and the tube containing it treated in exactly the same manner.

The next morning all the tubes were centrifuged until the supernatant fluid was quite clear. This was then pipetted off and tested for agglutinating power in each case—(1) against the emulsion which had been used for absorbing the serum, (2) against an emulsion of strain "Italian" 11, which was the strain used in preparing the serum. These results were compared with those obtained with the control serum. The emulsions used in these tests were diluted peptone-water cultures containing 1 in 1000 of formalin.

In Table III the agglutinating powers of these absorbed and unabsorbed sera are given, the emulsions used in the agglutination tests being prepared from the vibrio used for absorbing the serum.

TABLE III.

Strain.	Treatment of serum.	Dilutions of serum used in agglutinating tests. 1 in— 50	100	200	400	800	1600	3200	6400	Salt sol. control.
Italian 11 (homologous str.)	Unabs. dil. 1/10	+	+	+	+	S.+	tr.+	tr.	—	—
Ditto	1/5 dil. abs. with emuls. dil. 1/5	+	+	S.+	S.—	—	—	—	—	—
„	1/5 dil. abs. with undil. emuls.	+	S.	—	—	—	—	—	—	—
R.A.M.C. 2	Unabs. dil. 1/10	+	+	+	+	S.	tr.	—	—	—
„	1/5 dil. abs. with emuls. dil. 1/5	+	+	S.+	S.—	—	—	—	—	—
„	1/5 dil. abs. with undil. emuls.	+	S.	—	—	—	—	—	—	—
Galicia J.F. 7	Unabs. dil. 1/10	+	+	+	+	S.	—	—	—	—
„ „	1/5 dil. abs. with emuls. dil. 1/5	+	+	+	S.	—	—	—	—	—
„ „	1/5 dil. abs. with undil. emuls.	+	S.	—	—	—	—	—	—	—
Pasteur Inst. M.	Unabs. dil. 1/10	+	+	+	+	S.+	S.—	—	—	—
„ „	1/5 dil. abs. with emuls. dil. 1/5	+	+	S.	—	—	—	—	—	—
„ „	1/5 dil. abs. with undil. emuls.	+	S.	—	—	—	—	—	—	—
Pasteur Inst. P.	Unabs. dil. 1/10	+	+	+	S.+	S.—	—	—	—	—
„ „	1/5 dil. abs. with emuls. dil. 1/5	+	+	S.	—	—	—	—	—	—
„ „	1/5 dil. abs. with undil. emuls.	S.	tr.	—	—	—	—	—	—	—

In Table IV the agglutinating powers of the same absorbed sera in respect of an emulsion of strain "Italian" 11 are shown. This strain of *V. choleræ* was the one used in the preparation of the serum.

TABLE IV.

Strain.	Treatment of serum.	Dilutions of serum used in agglutinating tests. 1 in— 50	100	200	400	800	1600	3200	6400	Salt sol. control.
Italian 11 (homologous str.)	Unabs. dil. 1/10	+	+	+	+	S.+	tr.+	tr.	—	—
Ditto	1/5 dil. abs. with emuls. dil. 1/5	+	+	S.+	S.—	—	—	—	—	—
„	1/5 dil. abs. with undil. emuls.	+	S.	—	—	—	—	—	—	—
R.A.M.C. 2	1/5 dil. abs. with emuls. dil. 1/5	+	+	S.+	S.—	—	—	—	—	—
„	1/5 dil. abs. with undil. emuls.	+	S.	—	—	—	—	—	—	—
Galicia J.F. 7	1/5 dil. abs. with emuls. dil. 1/5	+	+.	+	S.	—	—	—	—	—
„ „	1/5 dil. abs. with undil. emuls.	+	S.	—	—	—	—	—	—	—
Pasteur Inst. M. 9	1/5 dil. abs. with emuls. dil. 1/5	+	+	S.	tr.	—	—	—	—	—
„ „	1/5 dil. abs. with undil. emuls.	+	S.	—	—	—	—	—	—	—
Pasteur Inst. P. 10	1/5 dil. abs. with emuls. dil. 1/5	+	+	+	S.	—	—	—	—	—
„ „	1/5 dil. abs. with undil. emuls.	+	S.	—	—	—	—	—	—	—

The results obtained in these experiments conclusively confirm the results shown by the simple agglutination tests, namely, that, at any rate with the strains of *V. choleræ* which had been collected, there were no serological differences. As these strains were obtained from widely different sources it is probable that *V. choleræ* forms, serologically, a sharply defined group.

Further experiments showed that the serum prepared by injecting rabbits with the strains of *V. choleræ* "Italian" 11, R.A.M.C. 2 and Galicia J.F. 7 had no power of agglutinating suspensions made from cultures of *V. paracholeræ* A or B (Mackie) when the emulsions were made by diluting peptone-water cultures and adding formalin to bring the concentration to 1 in 1000.

In view of these results it was somewhat surprising that so many authors had given divergent opinions as to whether certain vibrios were true *V. choleræ*. The following experiments were therefore carried out, in which, instead of employing all the essential portions of Dreyer's technique* in making the agglutination tests, the bacterial emulsions were made by suspending in salt solution the vibrios grown on agar for eighteen to twenty hours at 37° C., the emulsions being then reduced to a similar opacity.

In Table V the results obtained with the serum "Italian" 11 are shown. The readings were made after the tubes had been kept at 50° to 55° C. for two hours in the water-bath.

TABLE V.

Strain.	Dilutions of serum. 1 in—						
	50	100	200	400	800	1600	Control.
Italian 11 (homologous str.)	+	+	+	+	S.	—	—
R.A.M.C. 2	+	+	S.+	S.—	—	—	—
R.A.M.C. 4	+	+	+	+	S.+	S.—	—
R.A.M.C. 5	+	+	+	+	S.—	tr.	—
R.A.M.C. 6	+	+	+	S.	—	—	—
Galicia J.F. 7	+	+	S.	tr.	—	—	—
Pasteur Inst. M. 9	+	+	+	+	S.	—	—
Pasteur Inst. 8	+	+	+	S.+	tr.	—	—
Pasteur Inst. P. 10	+	+	+	+	S.	—	—
V. parachol. A	+	+	+	S.+	tr.	—	—
V. parachol. B	tr.	+	+	+	S.	tr.	—

On examining these results it is at once seen that they are very irregular compared with those obtained with emulsions made from broth cultures, true *V. choleræ* strains being agglutinated to titres varying from 200 to nearly 1600, and the two strains of *V. paracholeræ* up to a titre of about 400 in the case of the A strain and 800 in the B strain. When, however, the same serum was employed together with an emulsion made from a diluted and formolised peptone-water culture neither the A nor B strain of *V. paracholeræ* was agglutinated by any dilution between 1 in 25 and 1 in 1600. On other occasions, however, especially when the time that the agglutination tubes were kept in the water-bath was considerably prolonged, emulsions made from agar cultures gave results approaching very closely those obtained with emulsions made from broth cultures.

* An account of Dreyer's technique will be found in the Medical Research Council's Special Report Series, No. 51, London, 1920 (H.M. Stationery Office), p. 136.

These results emphasise the importance of using in all similar investigations emulsions made from broth cultures, as recommended by Dreyer, which have been diluted to a suitable opacity with salt solution and formolised to a concentration of 1 in 1000.

Having found that *V. choleræ* probably formed a compact serological group, cultures of a number of other vibrios were collected and tested to ascertain if any of them were agglutinated by serum made from a strain of *V. choleræ*.

Besides the two strains of *V. paracholeræ* A and B, cultures of *V. nasik* and the El Tor vibrios A, 4, 5, 10 and 35 were collected. These cultures were frequently subcultured both on solid and liquid media, and then emulsions were made by growing the organisms in broth for eighteen to twenty-four hours at 37° C., diluting to a convenient and equal opacity and adding sufficient formalin to make the final concentration equal to 1 in 1000.

The first organism to be tested was *V. nasik*. The results of the agglutination tests with the three sera prepared from the strains of *V. choleræ* "Italian" 11, R.A.M.C. 2 and Galicia J.F. 7 are shown in the following table:

TABLE VI.

Strain used to prepare serum.	Strain used to make emuls.	Dilutions of serum. 1 in—							
		50	100	200	400	800	1600	3200	Control.
Italian 11 . .	Italian 11 .	+	+	+	+	+	S.	—	—
„ . .	*V. nasik* .	—	—	—	—	—	—	—	—
R.A.M.C. 2 .	Italian 11 .	+	+	+	+	+	+	S.+	—
„ .	*V. nasik* .	—	—	—	—	—	—	—	—
Galicia J.F. 7 .	Italian 11 .	+	+	+	+	+	+	S.+	—
„ „ .	*V. nasik* .	—	—	—	—	—	—	—	—

These results show that emulsions of *V. nasik* are not agglutinated by the serum made from any of the strains of *V. choleræ*.

The next experiments were carried out with the El Tor vibrios and with *V. paracholeræ* A and B. In order to assist in subdividing these organisms into serological groups, a serum was prepared by inoculating a rabbit with a suitable dose of vaccine prepared from *V. paracholeræ* A.

Emulsions of *V. paracholeræ* A, even when made by diluting and formolising broth or peptone-water cultures, were *now* found to be agglutinated by sera prepared from strains of *V. choleræ* to a titre somewhat higher than similar emulsions of the homologous vibrio.

This property, which, as has been mentioned above, was completely absent when the culture was first obtained, only gradually developed after repeated subcultures had been made on fluid and solid media.

Once the property had been developed it remained constant, and the specificity, which was present when the culture was first obtained, was not regained either by repeated daily subcultures in fluid media or the passage by intraperitoneal and subcutaneous injection through a series of sixteen guinea-pigs, a proceeding which completely restored the virulence.

The first series of agglutination tests were carried out with a cholera agglutinating serum prepared by means of the strain *V. choleræ* "Italian" 11.

Table VII shows the results obtained with various organisms.

TABLE VII.

Strain.	Dilutions of serum. 1 in— 25	50	100	200	400	800	1600	3200	6400	12800	Control.
V. parachol. A . .	+	+	+	+	+	+	+	+	S.+	tr.+	—
V. parachol. B . .	—	—	—	—	—	—	—	—	—	—	—
V. El Tor A. . .	+	+	+	+	+	+	S.	0	0	0	—
V. El Tor 4 . .	+	+	+	+	+	+	+	S.—	tr.—	0	—
V. El Tor 5 . .	+	+	+	+	+	+	S.	0	0	0	—
V. El Tor 10 . .	—	+	+	+	+	+	+	0	0	0	—
V. El Tor 35 . .	—	—	—	—	—	—	—	0	0	0	—
V. chol. Italian 11 Control . . .	0	0	+	+	+	S.+	tr.	—	—	0	—

On examining this table it is seen that emulsions of El Tor vibrios A, 4 and 5 were agglutinated to a titre approximately the same as the homologous vibrio, while emulsions of *V. paracholeræ* A and the El Tor vibrio 10 were agglutinated to a titre considerably higher. The emulsions of *V. paracholeræ* B and the El Tor vibrio 35 were not agglutinated by any of the dilutions of the serum employed.

Absorption experiments were therefore carried out to ascertain if the El Tor vibrios A, 4, 5 and 10 belonged to the *V. choleræ* or to the *V. paracholeræ* A group, and also to ascertain if emulsions of *V. paracholeræ* A had acquired the property of absorbing the agglutinating power from a cholera agglutinating serum. In the following tables are shown the results obtained. The undiluted emulsions used in absorbing the serum contained 100,000 million vibrios per c.c., and equal volumes of the absorbing emulsion were added to an equal volume of the serum diluted 1 in 10.

In Table VIII the serum was prepared with the strain of *V. choleræ* "Italian" 11.

TABLE VIII.

Serum 1/10 dil. prepared from str. Italian treated with—	Strain used in the preparation of the emulsion used in the agglutination tests.	Dilutions of serum for agglutination test. 1 in— 100	200	400	800	1600	3200	6400	Control.
Salt sol. control . .	Italian 11 . . .	+	+	+	S.+	tr.	—	—	—
Abs. with undil. emuls. of *V. paracholera* A.	Italian 11 . . .	+	+	+	S.—	—	—	—	—
Abs. with emuls. of *V. parachol.* A dil. 1/5	Italian 11 . . .	+	+	+	S.+	tr.	—	—	—
Abs. with emuls. of *V. parachol.* A dil. 1/25	Italian 11 . . .	+	+	+	+	S.+	tr.	—	—
Abs. with undil. emuls. of V. El Tor A.	Italian 11 . . .	—	—	—	—	—	—	—	—
Abs. with emuls. of V. El Tor A dil. 1/5	Italian 11 . . .	—	—	—	—	—	—	—	—
Salt sol. control . .	V. El Tor A. . .	+	+	+	S.	—	—	—	—
Abs. with undil. emuls. of V. El Tor 5	Italian 11 . . .	—	—	—	—	—	—	—	—
Abs. with emuls. of V. El Tor 5 dil. 1/5	Italian 11 . . .	—	—	—	—	—	—	—	—
Salt sol. control . .	V. El Tor 5 . .	+	+	+	S.	—	—	—	—
Abs. with undil. emuls. of V. El Tor 4	Italian 11 . . .	—	—	—	—	—	—	—	—
Salt sol. control . .	V. El Tor 4 . .	+	+	+	S.+	—	—	—	—

On examining the results shown in this table it is seen that vibrios El Tor A, 4 and 5 absorbed from the cholera agglutinating serum the power to agglutinate emulsions of the *V. choleræ* "Italian" 11 which had been used to prepare the serum. These strains of the El Tor vibrios must, therefore, be considered to be strains of true *V. choleræ*. As regards *V. paracholeræ* A, although emulsions of this organism were found to have acquired the property of being constantly agglutinated by serum prepared with strains of *V. choleræ*, even a volume of the serum diluted 1 in 10 absorbed with an equal volume of emulsion containing 100,000 million vibrios per c.c. was only reduced in the agglutinating power by 30 per cent. When the absorbing emulsion contained 20,000 million vibrios per c.c. there was no reduction in the agglutinating power of the serum, and when the absorbing emulsion contained 4000 million vibrios per c.c. it was found actually to have acquired an increased agglutinating power towards emulsions made from the homologous strain of *V. choleræ*. (This last observation illustrates a somewhat uncommon phenomenon occasionally noticed when sera are absorbed with an organism belonging to a different species to that used in preparing the serum.) These observations show that although *V. paracholeræ* A had become agglutinable with sera prepared from *V. choleræ* this was not specific agglutination. Further confirmation of the difference in agglutinating properties of *V. choleræ* and *V. paracholeræ* A is also shown in Table IX, where it is seen that a serum prepared from *V. paracholeræ* A, although agglutinating emulsions of this organism in dilutions up to 1 in 3200, failed to agglutinate emulsions of *V. choleræ* even in dilutions of 1 in 100.

TABLE IX.

Serum dil. 1/10 prepared from *V. parachol.* A treated with—	Strain used in preparation of the emulsion used in the agglutination tests.	Dilutions of serum for agglutination tests. 1 in—							
		100	200	400	800	1600	3200	6400	Control.
Salt sol. control . .	*V. parachol.* A . .	+	+	+	+	S.+	S.	—	—
Salt sol. control . .	V. El Tor 10 . .	+	+	+	+	+	S.+	—	—
Abs. with undil. emuls. of V. El Tor 10	*V. parachol.* A . .	—	—	—	—	—	—	—	—
Abs. with emuls. of V. El Tor 10 dil. 1/5	*V. parachol.* A . .	tr.	—	—	—	—	—	—	—
Abs. with emuls. of V. El Tor 35 undil.	*V. parachol.* A . .	+	+	+	+	+	S.	—	—
Abs. with emuls. of V. El Tor 35 dil. 1/5	*V. parachol.* A . .	+	+	+	+	+	S.	—	—
No treatment . . .	*V. chol.* Ital. . .	—	—	—	—	—	—	—	—

Table IX also shows the results of absorbing a serum made by means of *V. paracholeræ* A in respect of its agglutinating power with emulsions of the El Tor vibrios 10 and 35. They indicate that the El Tor vibrio 10 completely absorbed the agglutinating power from the serum in respect of emulsions of *V. paracholeræ* A, so that El Tor vibrio 10 must be considered identical with *V. paracholeræ* A.

The El Tor vibrio 35, however, showed no absorption and must therefore be considered distinct from the *V. paracholeræ* A group. Unfortunately no serum had been prepared from *V. paracholeræ* B, so it was not possible to ascertain if this organism was identical with that vibrio.

SUMMARY.

The results obtained show that:

(1) All the strains of *V. choleræ* investigated belonged to one serological race, and as these strains came from widely different sources it is probable that there is only one serological race of *V. choleræ*.

(2) The advantage of using emulsions made by formolising broth cultures as recommended by Dreyer when comparing the agglutinating properties of a series of strains of the same organism is sharply brought out.

(3) After continued subculture on artificial media emulsions of *V. paracholeræ* A acquired the property of being agglutinated by cholera-agglutinating sera, but these emulsions had no power of absorbing the agglutinins for emulsions of the homologous vibrio. This property was not lost either by repeated daily subculture in fluid media or by passage through a series of guinea-pigs sufficiently numerous completely to restore the virulence of the organism.

(4) Of the El Tor vibrios, the strains known as A, 4 and 5 behaved both in their agglutinating and absorbing properties as true *V. choleræ* and should therefore be considered as belonging to this species. Strain 10 behaved in an exactly similar manner to *V. paracholeræ* A, being agglutinated by an anti-serum prepared from this organism and absorbing the specific agglutinins from the *V. paracholeræ* A anti-serum, and must therefore be considered as belonging to this species. *V. nasik*, *V. paracholeræ* B and the El Tor vibrio 35 had no serological relation with either *V. choleræ* or *V. paracholeræ* A and could not be classified.

THE GASTRIC AND RESPIRATORY RESPONSE TO MEALS.

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In previous communications [1 2] we have independently called attention to (*a*) the various types of gastric secretion and motility found in normal men (by T. I. B.), and (*b*) the alterations in the tension of alveolar CO_2 which follow the ingestion of a meal (by E. C. D.). A full account of the first subject will be found in an article written in conjunction with J. A. Ryle in the current number of *Guy's Hospital Reports*.[3]

In the present article we bring forward the results of work done in collaboration, in which the responses of a series of individuals to both tests may be compared; we believe that by this means we have produced corroboration of one another's findings, and that examination of the parallel results may throw some light on problems connected with gastro-intestinal secretion.

The subjects from whom the present results were obtained were, in two respects, selected ones:

(1) They were all men in good health, without symptoms or previous history of gastro-intestinal disease.

(2) Some of them were chosen for alveolar air examination because their gastric analyses had shown them to be variants from the more usual type.

For indications as to what proportion of such cases may be found among normal men, the reader is referred to the article by Bennett and Ryle mentioned above.

TECHNIQUE.

A. *Gastric Analysis.*—The method used was the fractional one introduced by Rehfuss [4, 5], the exact technique following closely that employed by Crohn and Reiss.[6]

The test-meal consists of a pint of thin oatmeal gruel, which gives a gastric response quantitatively slighter than that obtained with a bulkier meal of

higher protein content. Ryle's modification of the Einhorn duodenal tube(7) is swallowed by the fasting subject, and after all the "resting-juice" has been withdrawn, the meal is swallowed and samples are withdrawn every quarter-hour until the stomach is empty.

These samples are then analysed, the percentage of free hydrochloric acid and the total acidity being estimated in each case by titration with decinormal alkali, using dimethylamidoazobenzol and phenolphthalein as indicators. The disappearance of the meal from the stomach is estimated by noting the last sample in which starch, as tested for by iodine, is present.

B. *Estimation of alveolar carbon dioxide tension.*—It will be well to refer at this point very briefly to the results of an earlier investigation.(2) In a series of twelve normal persons the alveolar carbon dioxide pressure was found to show the following changes after a meal: (1) A rise of from 2 to 6 mm. within the first half or three-quarters of an hour; (2) a subsequent fall of about the same amount (2–6 mm.) below the original level; (3) a return to this level. In a man from whom the greater part of the stomach had been removed, the rise after a meal was very small in amount (0·4 to 0·8 mm.), while the subsequent changes were similar to those seen in a normal person. It seemed probable that the rise was associated with the secretion of gastric juice, and the subsequent fall with the later processes of digestion.

In the later observations on CO_2 tension which are dealt with in the present paper it seemed best to adhere to that form of meal with which previous experience had been gained, although this is different from the test-meal of gruel. After the subject had rested for half an hour samples of alveolar air were taken shortly before lunch, which consisted of beef, potatoes, bread and cheese; after the meal samples were taken every quarter or half-hour until the tension remained approximately constant, near the original level, in two successive samples. It was, of course, necessary to be sure that the changes in alveolar carbon-dioxide tension following upon breakfast were not still in progress: the subjects were instructed to take breakfast not later than 8 a.m., and the duplicate samples of alveolar air taken before lunch were separated by a quarter of an hour's interval so that the

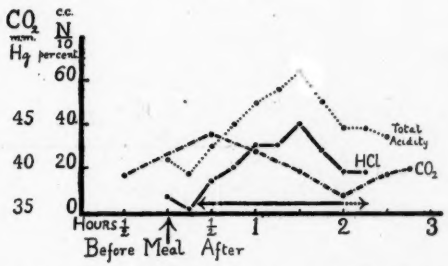

FIG. 1.—To show curves of gastric acidity and alveolar CO_2 tension in a subject of usual type (Case 1). The arrow in this and subsequent figures indicates the emptying time of the stomach, as shown by the duration of a positive iodine test for starch. A dotted portion of the arrow indicates a faint trace of starch only.

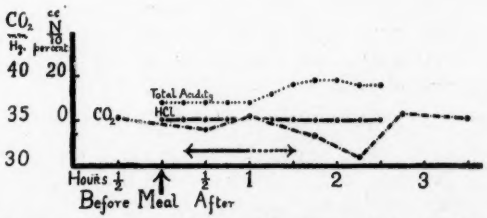

FIG. 2.—To show curves of gastric acidity and alveolar CO_2 tension in a subject of achlorhydric type (Case 10).

meal could be deferred if any distinct difference between these two samples were found. All samples were taken by the Haldane-Priestley method, each point on the curve being the mean of two samples—an expiratory and an inspiratory.

RESULTS.

Each of the four normal types previously described (1) was examined.

(1) *The usual type* (Fig. 1, Case 1).—The curve of gastric HCl percentage here shows a steady rise after the test-meal, reaching a height between 20 and 50 c.c. of N/10 per cent., and then falling as the regurgitation of alkaline duodenal contents occurs at the end of gastric digestion.

The tension of alveolar CO_2 shows the rise followed by a fall, which has already been observed and ascribed to gastric secretion and a later secretion in lower portions of the alimentary tract.

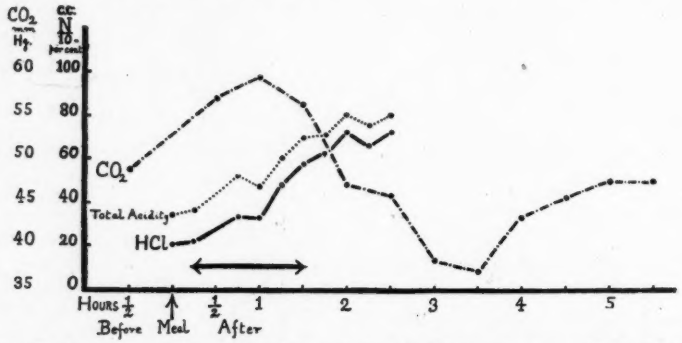

FIG. 3.—To show curves of gastric acidity and alveolar CO_2 tension in a subject of "hyperchlorhydric" or "hypersecretory" type (Case 11).

We have observed curves in all ways similar to these in eight cases examined by the double method.

(2) *The type with low gastric acidity or achlorhydria* (Fig. 2, Case 10).—The example illustrated is from a man, apparently quite healthy, who at no time shows any free HCl to be present in his stomach. Comment on the gastric analysis is superfluous; the curve of alveolar CO_2 tension is strikingly similar to those obtained from a patient who had had a complete gastrectomy, which have been published elsewhere.(2)

(3) *The type with gastric hypersecretion or hyperchlorhydria* (Figs. 3 and 4).—The first example illustrated (Fig. 3) is from a healthy man (Case 11), who shows hyperchlorhydria in rather an extreme degree. At the end of an hour the free HCl reaches 50 c.c. N/10 per cent., whereas the average in a large series is not higher than about 30 c.c.; more striking still is the fact that after this point the acidity continues to rise, probably being helped by the absence of the duodenal regurgitation which occurs in the majority of normal persons.

The alveolar CO_2 tension in the same subject shows a remarkable range,

rising no less than 10 mm. after the meal and then falling to a similar extent before returning to its original level.

The second example (Fig. 4, Case 12) of this type is equally remarkable; the gastric analysis does not at first glance reveal very remarkable figures, as the acidity does not rise much beyond 50 c.c. of N/10 per cent., but there is the same absence of fall at the end of the curve that was present in the previous subject. It was observed, however, that in this subject 140 c.c. of juice were present in the resting stomach, whereas the usual figure is about 40 c.c.; and at the end of three hours a little of the test-meal was still present

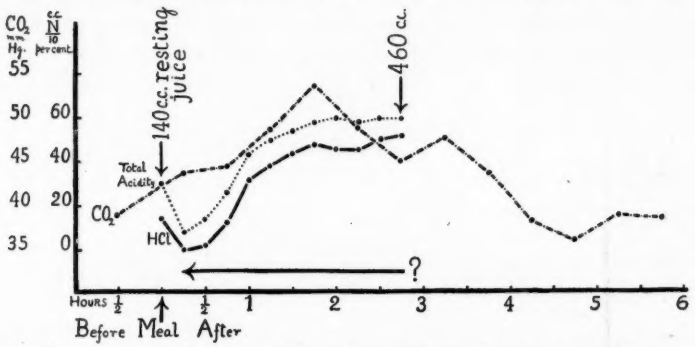

FIG. 4.—To show curves of gastric acidity and alveolar CO_2 tension in a subject of "hypersecretory" or "hyperchlorhydric" type (Case 12).

and no less than 460 c.c. of gastric contents were removed. In fact the 50 c.c. of N/10 per cent. present in the stomach really represented an enormous secretion of gastric juice. We were therefore not surprised to find that this individual gives a rise of 15 mm. in alveolar CO_2 tension after a meal, which is greater than that of our previous subject No. 11. Further comment on these findings will be found in a later section of this paper.

(4) *The rapid type* (Fig. 5, Case 7).—The subjects of whom an example is here shown have a normal gastric acidity but a remarkably rapid gastric cycle; exactly parallel results have been obtained in the alveolar CO_2 estimations (Cases 7 and 8). In many of these subjects samples must be taken at very frequent intervals if the variations are to be observed. The stomach has emptied itself after ¾ hour, whereas in the more usual type this process takes from 1½ to 2½ hours.

These three less usual types are shown together in Fig. 6.

In Table I the figures are given from twelve of the cases we have investigated together; they include only a certain propor-

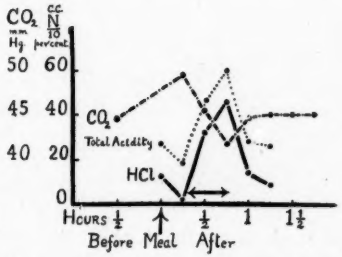

FIG. 5.—To show curves of gastric acidity and alveolar CO_2 tension in a subject of "rapid" type (Case 7).

tion of our results, and are published merely to illustrate the type of results of which the curves in the text are examples.

In Table II will be found corresponding figures (*a*) to convert c.c. N/10 per cent. into the true percentage of HCl, and (*b*) to convert tension of alveolar CO_2 in mm. Hg. into actual percentage of CO_2, this latter table being applicable when the atmospheric pressure for the day is 760 mm. Hg.

COMMENTARY.

Further investigation of these cases is in progress, and we hope at a later date to publish, in conjunction with E. L. Kennaway, the results of urinary and blood examinations in the same series. Until the complete figures are before us we prefer to reserve full discussion of our findings. To three things, however, we wish now to draw attention:

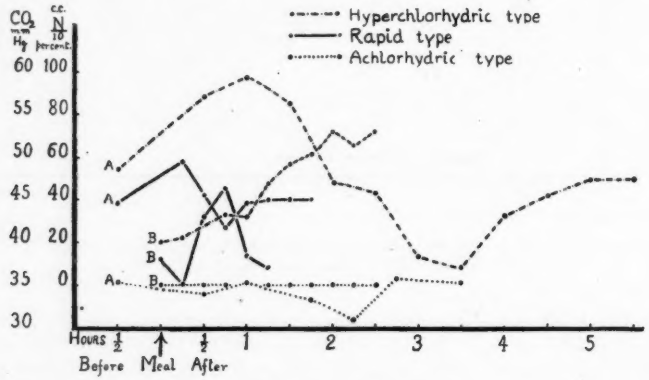

FIG. 6.—To contrast the curves of the three less usual types:
Example shown of hyperchlorhydric type is Case 11.
" " rapid " " 7.
" " achlorhydric " " 10.
Curve A: Alveolar CO_2, mm. Hg. Curve B: Free HCl, c.c. $\frac{N}{10}$ per cent.

(1) To the evidence in Fig. 2 of normal secretion below the pylorus, even in the absence of gastric HCl. In a recent article on achylia gastrica,(8) one of us summarised the functions of the gastric HCl as follows: "It is a useful but merely accessory agent for protein digestion, a useful but accessory agent for controlling the action of the gastric sphincters, an important but not essential means of diminishing bacterial growth in the intestines."

The immunity from symptoms of many persons with achlorhydria has long been observed; the curve of alveolar CO_2 tension in these cases supports the belief that the secretions in lower portions of the alimentary canal can proceed normally in the absence of any excitation due to hydrochloric acid.

(2) To the differences in alveolar CO_2 tension in different subjects during the "resting" period of digestion. In Fig. 6 will be found plotted together the results from several different types; it will be observed that those whose

TABLE I

CASE No.	BEFORE MEAL: ½ HOUR BEFORE MEAL	BEFORE MEAL: JUST BEFORE MEAL	AFTER MEAL: ¼ HOURS	½	¾	1	1¼	1½	1¾	2	2¼	2½	2¾	3	3¼	3½	4	4½	5	5½	
1. A		7	0	14	20	29	30	40	28	18	18	18									AVERAGE TYPE VIDE FIG. 1.
1. B		24	18	30	40	51	56	64	50	38	40	42									
1. C	39·2			43·8		41·9		39·6		36·9		39·2	39·2								
2. A		0	0	4	16	32	40														AVERAGE TYPE RATHER RAPID
2. B		10	8	20	34	46	54														
2. C	39·8			45·3		36·7		34·5		40·4		41·2									
3. A		26	0	4	10	22	20	22	2	16	10	12									AVERAGE TYPE
3. B		38	10	16	24	34	38	40	14	26	26	22									
3. C	38·4			40·5		38·6		36·3		37·8		38·6									
4. A		14	2	18	28	18	24	22	24	22	14	10									AVERAGE TYPE
4. B		26	20	34	44	36	42	40	42	40	24	22									
4. C	38·9			44·9		41·3		37·8		34·7			38·9	38·9							
5. A		0	0	0	12	16	16	20	14	10	15	20									AVERAGE TYPE
5. B		5	6	9	25	29	33	42	34	27	36	30									
5. C	39·2		42·2	42·6		42·6		42·2		40·3		33·9		39·9	39·6						
6. A		0	0	0	9	12	16	18	18	18											AVERAGE TYPE
6. B		16	6	11	29	34	36	36	33	33											
6. C	40·1		42·4	41·6		43·2		40·1		38·2		39·9	40·4								
7. A		12	0	32	46	12															RAPID TYPE VIDE FIG. 5
7. B		34	16	44	64	25															
7. C	44·7		49·5	45·5	41·7	44·7	45·1	45·1	45·1												
8. A		0	0	6	20	30															RAPID TYPE
8. B		11	14	24	31	44															
8. C	39·1		42·8	38·6	32·5	37·5	38·2	39·1	39·1												
9. A		0	0	0	0	0	0	0													ACHLORHYDRIC TYPE
9. B		6	6	6	8	8	6	6													
9. C	43·6		44	43·6		38·2	38·2	39·3	44·3	43·5		43·6									
10. A		0	0	0	0	0	0	0	0	0	0	0									ACHLORHYDRIC TYPE VIDE FIG. 2
10. B		8	8	8	8	8	12	16	18	18	16	16									
10. C	35·3			33·9		35·3			33·2		30·9		35·7	35·3							
11. A		20	22	?	33	32	48	57	62	72	66	72									HYPERSECRETION TYPE VIDE FIG. 3
11. B		34	36	?	53	46	60	67	69	80	75	80				SEPARATED BY ½ HR INTERVALS					
11. C	48·6			57·0		59·3		56·2		47·1		45·9		38·4		37·1	43·2	45·5	47·4	47·4	
12. A		14	0	2	12	32	38	44	48	46	46	50	52								HYPERSECRETION TYPE VIDE FIG. 4
12. B		30	8	14	26	44	50	54	58	60	58	60	60		SEPARATED BY ½ HR INTERVALS						
12. C	38·8		43·6		44·5		48·7		53·6		49·1		45·3		47·9	44·1	38·4	36·1	39·2	38·8	

A = FREE HCl EXPRESSED IN CC $\frac{N}{10}$ PER CENT.
B = TOTAL ACIDITY IN CC $\frac{N}{10}$ PER CENT.
C = ALVEOLAR CO_2 TENSION IN MM.

percentage of HCl later reaches a high figure show a high alveolar CO_2 tension, even before gastric digestion has begun; the converse is seen in cases of achlorhydria. The explanation of this is that gastric secretion, like gastric peristalsis, is a more continuous process than has been usually believed, the cycle of secretion that follows a meal being merely the accentuation of a process that continues in a much slighter degree at other times; much light has been thrown on this mechanism by the recent work of Carlson.(9)

(3) To the necessity for revising existing views as to the function of the respiratory centre.

At the present stage there does not seem to us to be any purpose in considering how far subjects such as the two with hyperchlorhydria, whose alveolar CO_2 tension ranges over as much as 20 mm. in the course of a few hours, are to be regarded as normal or pathological. We believe it to be a new observation that any respiratory centre, normal or abnormal, allows rapid changes of such magnitude to occur. It is evident that the centre does not maintain the CO_2 tension at a constant level; it seems rather to produce such variations in this tension as are appropriate to maintain some other factor constant, and in some persons these variations have to be very considerable. If the H-ion concentration of the blood is to be kept approximately constant these large fluctuations in alveolar CO_2 tension are bound to occur. For instance, when free hydrochloric acid is formed from the neutral body fluids and secreted into the stomach, an equivalent quantity of base must be left behind which will disturb the proportion $H_2CO_3/NaHCO_3$ upon which the reaction of the blood depends. It is then necessary to restore the balance by retaining carbon dioxide; if the amount of gastric acid secreted has been considerable as in our subjects 11 and 12 the lung ventilation may have to be lessened till the CO_2 tension has risen by as much as 10 mm. (Figs. 3 and 4).

TABLE II

CONVERSION OF $\frac{N}{10}$ NaOH PER CENT. INTO PERCENTAGE OF HCl.

cc $\frac{N}{10}$ NaOH	10	20	30	40	50	60	70	80	90
HCl PER CENT	·036	·073	·109	·146	·182	·219	·255	·292	·327

CONVERSION OF ALVEOLAR CO_2 TENSION AT 760 MM OF MERCURY INTO CO_2 PERCENTAGE.

CO_2 PER CENT	4	4·5	5	5·5	6	6·5	7
CO_2 TENSION	30·4	34·2	38·0	41·8	45·6	49·4	53·2

The respiratory centre, then, does not maintain a constant alveolar CO_2 tension. It may appear to do so if observations are made when no active digestion is in progress, because CO_2 is at these times by far the most abundant substance of acid or alkaline reaction which is entering or leaving the blood, and the maintenance of its tension at a constant level is then just what is required, in conjunction with the activity of the kidney, to preserve the ratio $H_2CO_3/NaHCO_3$ at its proper amount. That the alveolar CO_2 tension is preserved constant no longer than is suitable for the needs of the body has, of course, long been known in the case of acclimatisation to high altitudes; the observations brought forward in this paper show further that considerable variations occur as the result of an ordinary meal.

SUMMARY.

(1) Studied by means of gastric analysis, and by estimation of alveolar CO_2 tension, normal men are found to fall into four distinct classes.

(2) The results obtained by the two methods are found to confirm one another.

(3) Cases showing achlorhydria during gastric analysis show a curve of alveolar CO_2 tension similar to that obtained in patients following gastrectomy.

(4) Such cases show a fall in alveolar CO_2 tension at a later period, indicating normal secretion during the later processes in digestion.

(5) Cases of hyperchlorhydria in healthy men have sometimes been found to show changes in alveolar CO_2 tension following meals which exceed what have previously been considered physiological limits.

(6) Some normal cases occur in whom the processes of upper alimentary digestion are so rapid as to be only demonstrable by observations taken at very short intervals.

(7) Cases which yield a high gastric acidity during digestion are usually found to show a relatively high tension of alveolar CO_2 even during the "resting" phases of the stomach; in cases of achlorhydria the reverse is seen.

We wish to acknowledge our indebtedness to all those gentlemen, students of Guy's and the Middlesex Hospitals, who have so willingly given their time, often at great inconvenience, to serving as subjects for these experiments.

We also wish to acknowledge the constant and critical help of Dr. E. L. Kennaway.

The respiratory observations made in this work form part of a report to the Medical Research Council.

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AN INQUIRY INTO THE DISTRIBUTION OF THE BLOOD GROUPS IN PATIENTS SUFFERING FROM "MALIGNANT DISEASE."

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The following is a report of an investigation which is being carried out in the bacteriological laboratories of the University of St. Andrews, the object of which is to determine the distribution of the blood groups in individuals known to be suffering from "malignant disease."

The number of cases so far dealt with—fifty—is not sufficiently large to justify any definite conclusions being drawn, and all that is intended is to call attention to one or two features which have presented themselves in the course of the inquiry.

We know that blood-grouping follows Mendelian laws, but we are ignorant of what really constitutes "group": and we are also ignorant of the mechanism of hæmagglutination. Again, we do not know all the factors that determine the occurrence of malignant disease, but there is more than a suggestion that a hereditary factor, as distinct from inherited infection, plays some part in its causation. In view thereof, it was decided to carry out the investigation herein described.

Figures relating to the blood groups of persons suffering from tuberculosis, syphilis and tetanus—infective diseases of known ætiology, and susceptible to investigation by immunological methods—are included in the series. These serve the double purpose of controlling the results obtained in cases of malignant disease, and of showing that susceptibility to the infective maladies is in no way related to the blood groupings.

Should it ultimately be proved that individuals belonging to certain of the blood groups are more susceptible to malignant neoplasms than those belonging to others, a stimulus is given to inquire into the innate qualities that may be associated with susceptibility to malignant disease.

Under the term "Malignant disease" we have included cases of carcinoma, sarcoma, rodent ulcer and leukæmia, this last condition being regarded provisionally as a sarcoma of the hæmopoietic system. In the majority of cases of the present series the clinical evidence has been verified by post-mortem

and microscopical examination, and all cases in which the Wassermann reaction was positive have been excluded from the series.

The routine method was to test the patient's corpuscles against sera known to belong to Groups II and III, and the converse reaction, *i. e.* testing the serum of the patient under examination against corpuscles of known type, was only performed where any doubt as to the blood group arose, or as a confirmatory test in those cases where no agglutination was observed—that is, in all cases of Group IV. In selecting sera for test purposes, it is important that only those be employed which give specific results when tested by examination of a sampling of a general healthy population, and which give, when so tested, an approximately correct statistical result.

As a rule there was no difficulty in determining when agglutination had occurred, but it was considered advisable to set a time limit, and if agglutination was not obvious with one or other within 60 seconds when Group II and Group III sera were used, the patient was regarded provisionally as belonging to Group IV. Sometimes there was a tendency to rouleaux formation, and this may be mistaken for a positive result, and clotting may also produce an appearance simulating agglutination, but by the adoption of a time limit this latter danger may be eliminated.

TABLE I.—*Showing Distribution of Blood Groups in Fifty Cases of Malignant Disease.*

No.	Sex.	Age.	Disease.	Group.
1	M.	58	Epithelioma tongue	I.
2	M.	43	Carcinoma head of pancreas	I.
3	M.	39	Carcinoma head of pancreas	I.
4	F.	54	Carcinoma cervix uteri	I.
5	F.	59	Carcinoma breast	I.
6	F.	80	Rodent ulcer nose	I.
7	M.	60	Epithelioma bladder	II.
8	F.	37	Carcinoma sigmoid	II.
9	M.	64	Rodent ulcer nose	II.
10	M.	62	Carcinoma rectum	II.
11	M.	60	Carcinoma colon	II.
12	F.	52	Scirrhus breast	II.
13	F.	56	Carcinoma stomach	II.
14	M.	68	Sarcoma testicle	II.
15	F.	44	Malignant ovarian cyst; carcinoma stomach	II.
16	F.	58	Carcinoma sigmoid	II.
17	F.	62	Carcinoma rectum	II.
18	M.	60	Carcinoma sigmoid	II.
19	M.	57	Carcinoma pylorus	II.
20	M.	24	Sore on forearm, flat-celled epithelioma	II.

No.	Sex.	Age.	Disease.	Group.
21	M.	49	Carcinoma colon	III.
22	M.	67	Carcinoma colon	III.
23	M.	40	Carcinoma jaw	III.
24	M.	60	Carcinoma pylorus	III.
25	F.	70	Carcinoma stomach	III.
26	M.	83	Epithelioma tongue	III.
27	F.	46	Carcinoma head of pancreas	III.
28	M.	46	Carcinoma larynx	III.
29	F.	22	Myeloid leukæmia	III.
30	F.	20	Malignant tumour thyroid	III.
31	M.	47	Carcinoma stomach	III.
32	F.	44	Cylinder - celled epithelioma, sacral region	III.
33	F.	84	Rodent ulcer	III.
34	F.	38	Carcinoma œsophagus	III.
35	F.	57	Carcinoma cervix uteri	III.
36	F.	62	Carcinoma cervix uteri	III.
37	M.	54	Carcinoma colon	IV.
38	M.	49	Carcinoma rectum	IV.
39	F.	66	Carcinoma stomach	IV.
40	F.	72	Carcinoma rectum	IV.
41	M.	66	Rodent ulcer nose	IV.
42	F.	20	Myeloid leukæmia	IV.
43	M.	50	Flat-celled epithelioma nose	IV.
44	F.	24	Carcinoma rectum	IV.
45	F.	51	Scirrhus breast	IV.
46	F.	58	Carcinoma rectum	IV.
47	M.	55	Carcinoma stomach	IV.
48	M.	66	Carcinoma jaw	IV.
49	M.	40	Sarcoma testicle	IV.
50	F.	45	Carcinoma pylorus	IV.

In Table II these results are compared with those obtained in examining a similar number of specimens from (1) a normal population; (2) cases of pulmonary tuberculosis; (3) cases of active syphilis; (4) cases of tetanus. For information concerning these last I am indebted to Dr. W. J. Tulloch.

TABLE II.—*Showing a Comparison between the Incidence of the Blood Groups in Malignant Disease with that in Other Conditions.*

	Per cent. in 50 normal.	Per cent. in 50 cases of tuberculosis.	Per cent. in 50 cases of active syphilis.	Per cent. in 25 cases of tetanus.	Per cent. in 50 cases of malignant neoplasm.
Group I	4	4	4	4	12
Group II	38	34	36	32	28
Group III	12	12	12	16	32
Group IV	46	50	48	48	28

From the above figures it appears that individuals belonging to all four blood groups are liable to suffer from malignant disease. It would seem, however, that Groups I and III are peculiarly susceptible to the various forms of malignant new growth, while Group IV is more stable or resistant in this respect. The figures are insufficient to permit of any suggestion being offered concerning the susceptibility of Group II.

These findings are borne out from the clinical aspect of the cases, for speaking generally, carcinoma or sarcoma occurring in Groups I or III of the present series proved to be of a severe kind and the course rapid; on the other hand, in the case of Groups II and IV, especially Group IV, the tumour was usually of low malignancy and the duration of the disease long.

These results are published in the hope that workers in other parts may be tempted to carry out the blood grouping of cases of malignant disease so that an adequate statistic may soon be available.

In conclusion, I should like to express my indebtedness to Dr. Tulloch for many excellent suggestions and assistance, and also to Prof. L. R. Sutherland for supplying me with pathological reports on the cases.

To Dr. John Taylor and Dr. G. R. Ross, I owe a special debt of gratitude as they were the willing sources of my type sera. And lastly I have to thank the Superintendent and Consultant Staff of the Royal Infirmary, The Royal Victoria Hospital and King's Cross Hospital, Dundee, for permission to have access to the patients under their care.

CONCLUSIONS.

The blood group of 50 cases of malignant disease has been determined, and the incidence of each compared with that in 125 cases of other diseases and 50 normal persons.

It is found that, while persons belonging to all four groups are liable to malignant disease, those in Groups I and III appear to be peculiarly susceptible and the clinical type of disease is generally speaking more malignant.

THE INFLUENCE OF BILE-SALTS UPON GASTRIC FUNCTION.

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From the Physiological Laboratory, St. Mary's Hospital, London.

Received for publication January 13th, 1921.

In certain operations performed upon man for some gastric disorders the bile is allowed free access to the stomach. An investigation was undertaken to determine whether any alterations in its motor or secretory functions take place as the result.

In the first place digestion in the stomach of the cat was inquired into. It was found that digestion, although inhibited in its onset, would take place when the animal was under the influence of urethane. It is true that such digestion is not quite normal, as the reflex excitation of gastric juice secretion through the higher senses is eliminated. It was, indeed, an investigation of the second chemical phase of digestion of Pawlow. But therein resides a certain merit in the method, for the disturbing effect of higher centre activity is removed, and with it one liability to error.

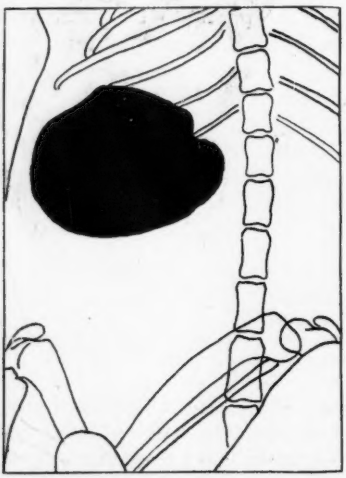

FIG. 1.

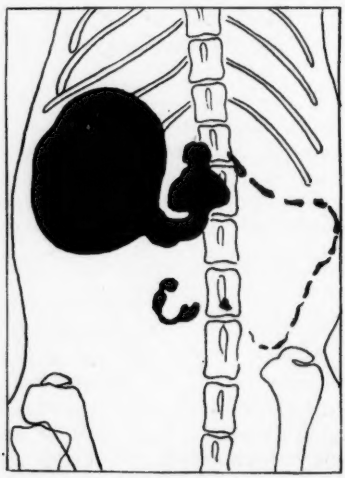

FIG. 2.

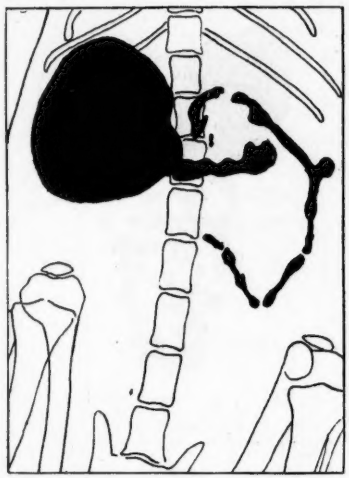

FIG. 3.

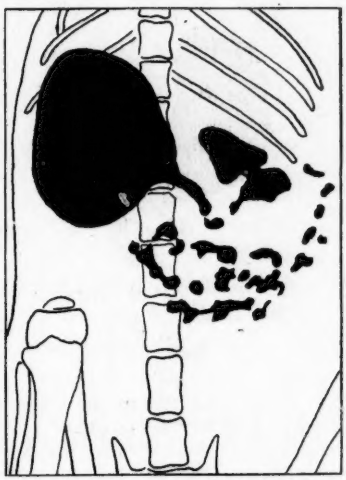

FIG. 4.

Experiment 1.—7/12/20: Tabby cat, weight, 3·5 kgrm. approximately; 4 grm. urethane subcutaneously. The following test-meal was administered by stomach-tube:

Rolled oats (1 oz. to 1 pint of water, boiled and filtered) . . .	50 c.c.
Barium sulphate . .	7·5 grm.

Skiagrams were taken 10 minutes, 25 minutes, 1 hour 20 minutes, 3 hours and 4 hours after the administration of the meal. Figs. 1 to 5 are tracings from the photographs. At the end of 4 hours there is still a considerable quantity of food in the stomach.

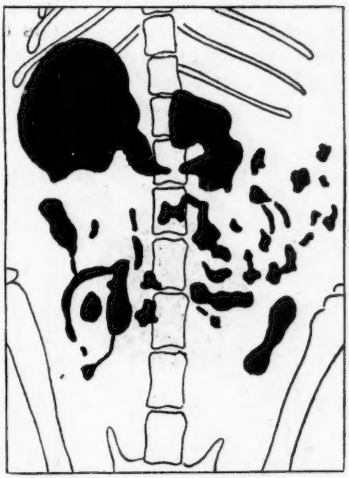

FIG. 5.

Experiment 2.—13/12/20: Same cat as in Experiment 1; 4 grm. urethrane subcutaneously. A similar meal given, with the addition of 0·175 grm. sodium taurocholate. Skiagrams were taken 7 minutes, 20 minutes, 35 minutes and 65 minutes after administration. Figs. 6 to 9 are the tracings. It will be seen that the stomach began to empty early, and the whole meal had passed the pylorus in a little over 1 hour.

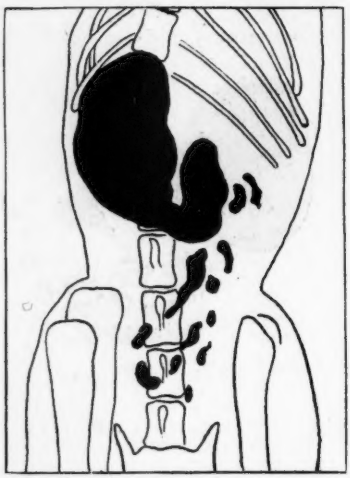

FIG. 6.

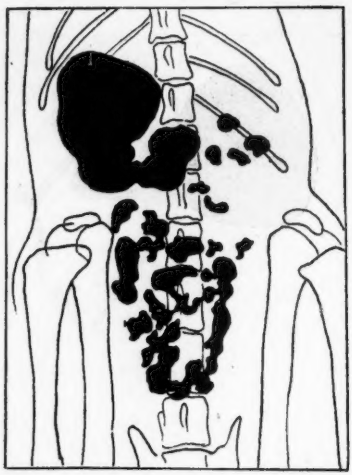

FIG. 7.

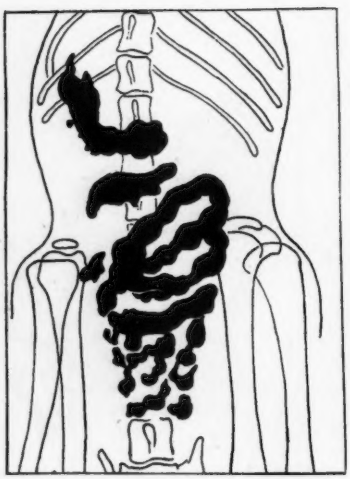

FIG. 8.

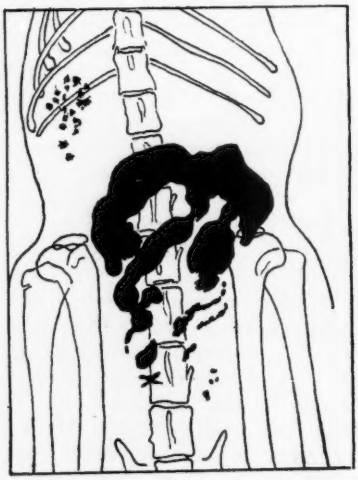

Fig. 9.

Experiment 3.—21/11/20: Black cat; 4 grm. urethane subcutaneously. Similar meal to that in Experiment 1. Screen observations.

Time, 12.30: Meal administered.

2 p.m.: None had passed the pylorus.

3 p.m.: About one-half of meal in the small intestine.

4 p.m.: Stomach almost empty.

Experiment 4.—28/11/20: Black cat; 4 grm. urethane subcutaneously. Similar meal to Experiment 2 administered. Skiagrams taken.

Time, 12 noon.: Meal administered.

12.10 p.m.: Food beginning to pass pylorus.

12.15 p.m.: Moderate quantity has passed into intestine.

12.30 p.m.: Considerable quantity in intestine.

1 p.m.: About one-half of meal has left stomach.

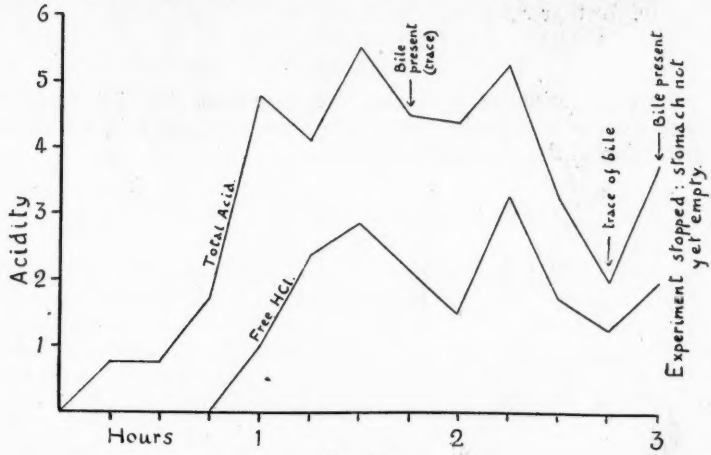

FIG. 10.

Stomach emptied some time between 1 p.m. and 1.55 p.m. No screen examination made between these times. Stomach quite empty at 1.55 p.m.

From these experiments it seems justifiable to conclude that the motor power of the stomach is greatly increased by the addition of sodium taurocholate to the gastric contents, and thereby a rapid evacuation takes place.

Corroborative evidence was obtained by employing the fractional test-meal method of investigation in a normal man.

Experiment 5.—20/11/20: C. A. P—, normal man. On an empty stomach a test-meal of 1 pint of gruel (rolled oats 1 oz. to water 1 pint) taken. Fractional estimation of stomach contents. Fig. 10 is the curve.

Experiment 6.—27/11/20: Same man as in Experiment 5. Same meal taken.

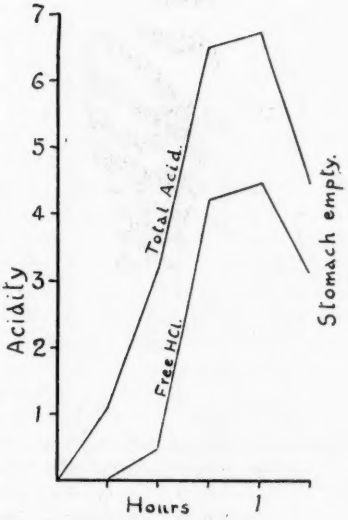

FIG. 11.

Then 1 grm. sodium taurocholate inserted into stomach down tube. The fractional curve is shown in Fig. 11.

From these experiments it will be seen that in a normal man also a slight addition of sodium taurocholate was followed by a rapid evacuation of the test-meal. The curve in Fig. 11 also shows a more rapid rise of acidity and an ultimate higher level. Whether or not this is a regular result and whether there is any alteration in digestive power of the juice secreted is at present the subject of further research.

CONCLUSIONS.

It is shown experimentally in cats and man that the addition of a small quantity of sodium taurocholate to a test-meal is followed by an abnormally rapid evacuation of the stomach contents.

THE ÆTIOLOGY OF RICKETS: AN EXPERIMENTAL INVESTIGATION.

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From the Institute of Physiology, University of Glasgow.

Received for publication December 2nd, 1920.

THE present investigation is a continuation of the experimental study of the ætiology of rickets which has been carried on in this laboratory since 1907, first by Leonard Findlay and later by Findlay, Noël Paton and Watson.

Since the publication of our last paper (1) some records of similar experimental work by Mellanby have appeared (2), and upon these a series of far-reaching conclusions have been formulated and published by the Vitamine Committee of the *Medical Research Council.*(3)

This report states "that rickets is a deficiency disease due to diets which are unbalanced in that they contain too little of those substances rich in an anti-rachitic factor and too much of those substances deficient in this respect. The anti-rachitic has, in many respects, a similar distribution to the fat-soluble A factor, and is possibly identical with this substance."

An examination of Mellanby's records shows that he reports none of the experiments upon which he bases the statement that "he cleared the way for his dietary experiments by a series of experiments to show that other factors were not operative." It is therefore impossible to estimate their value.

As to the influence of the dietary factors, he has recorded observations upon thirty-five pups, eleven normal and twenty-four which developed rickets. Presumably the experiments selected for publication were not the least convincing of his series. When the records are examined it is found that the dogs grouped together *were not always of the same litter.* Now, to contrast animals of different litters is, as we have stated before, wholly unjustifiable, since the susceptibility of different strains to rickets is notoriously different. Further, in his groups the pups were not always killed at the same age, and yet the calcium content of the bones is compared, although, as is known, this varies with age.

In this small series of observations no less than thirteen substances were tested and conclusions drawn as to which of these allows and which prevents the onset of rickets.

Our previously published work seems to show that such conclusions are unwarranted.

Findlay in 1908 (4) gave the result of his observations on the influence of diet and of confinement, from which he then came to the conclusion that the latter is the main factor in the development of rickets. That the condition developed was actually rickets was demonstrated by his histological examination of the bones, and the diagnosis was confirmed by Schmorl of Dresden, to whom the specimens were submitted.

Our observations published in 1918 were made upon seventeen puppies belonging to two litters. The main object was to get rickety pups and sound pups of the same litter and of the same age for chemical examination. But a test was also afforded of the relative influence of diet and open-air life.

From each litter two puppies were sent to the country, kept in a large yard and fed upon oatmeal porridge and skimmed milk, so that each had about 2·8 grm. of milk-fat per day—0·7 grm. per kilo. at the end of the experiment. Those kept in the laboratory had whole milk, containing, during the first period of their growth, about 3 grm. of butter-fat per day, and during the later period 6 grm.—about 2·4 grm. per kilo. at the end of the experiment.

In addition to this, three of the latter had butter, *not margarine*, in an amount to yield, along with the milk, 14 grm. of milk-fat *per diem*.

The puppies kept in the country on only 2·8 grm. of milk-fat remained absolutely normal, whereas all the puppies kept in the laboratory developed rickets in spite of the ample supply of milk-fat (from 3 up to 6 grm.), and one of those receiving butter (in all 14·5 grm. of milk-fat) became most markedly affected.

It has been suggested that puppies kept in the country get an anti-rachitic or fat-soluble substance by eating grass. But they did not eat grass, and further, the carnivorous alimentary canal does not digest grass even when swallowed. It has also been argued that confinement may so depress digestion and metabolism that the fat-soluble substance is not liberated or absorbed from the food, but there was no indication of disturbed digestion in the animals kept in the laboratory. Mellanby maintains that pups may be reared in confinement free from rickets if the diet contains an adequate supply of some anti-rachitic substance.

These observations seem to negative the view that rickets is due to the absence of a hypothetical anti-rachitic substance associated with milk-fat.

In this experiment the pups were given a very large amount of oatmeal and the energy intake was superabundant.

PRESENT INVESTIGATION.

Litters V and VI.

During the early months of 1920 observations were made upon two litters of pups with a more limited energy supply in the food.

The object of the experiment was to test the possibility of rearing dogs free from rickets in confinement on a diet rich in milk-fats with which the Vitamine Committee (3) maintain that an anti-rachitic accessory factor or vitamine is associated, and on one poor in these fats supposed to contain this hypothetical vitamine.

One observation on the effect of substituting dried milk for fresh milk was also made.

The diet consisted of a weighed quantity of oatmeal made into porridge, with, in some cases, a measured amount of whole milk and in others a similar amount of separated milk, and in one case an equivalent amount of whole cream dried milk (Cow and Gate brand).

The composition of the milks was as follows:

Whole milk.

	Grm.	Calories.
Fat	3·0	27
Protein	3·5	32·9
Lactose	4·5	

59·9 per 100 grm.

Separated milk.

	Grm.	Calories.
Fat	0·33	2·1
Protein	3·5	32·9
Lactose	4·5	

35 per 100 grm.

Dried whole cream milk (Cow and Gate), see p. 79.

The same weight of oatmeal and of milk, whole or separated, was given to each animal.

The first litter consisted of three pups which were put upon the diet at the age of ten weeks. One, A, had 30 grm. and later 45 grm. of oatmeal as porridge, with 250 c.c. of whole milk; the second, B, had the same amount of oatmeal with 250 c.c. of skimmed milk; while the third had the same quantity of oatmeal with milk made by dissolving 37 grm. of full cream dried milk (Cow and Gate brand).

The pups of this litter were killed at the age of twenty weeks. (Table I, Litter V, p. 86.)

The second litter consisted of six pups, which were put upon the diet at the age of five weeks. Two of these were put upon the same diet as A in the last series; a third dog was put upon this diet for a month, when the milk was reduced to 150 c.c.

Another pup was put upon the same diet as B in the last series, and two others were put upon this diet for a month, when the milk was reduced to 150 c.c.

In both litters the oatmeal was increased from 30 grm. to 45 grm. at the end of a month.

Four of the pups of the second litter were killed at fifteen weeks old. (Table I, Litter VI, p. 86.)

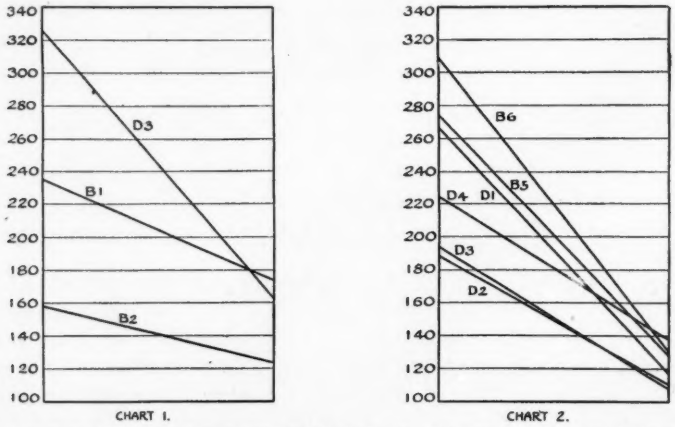
In our experience, and apparently in that of Mellanby, rickets develops before the fifteenth week, and if the pup reaches that age without manifesting signs of the disease, it usually remains free from it, however it may be treated. We generally found that if rickets was going to develop it became manifest at about the twelfth week of life.

One pup, D 1, on 250 c.c. of whole milk, which at fourteen weeks of age showed slight signs of rickets by X-ray photography, and one on 150 c.c. of skimmed milk with well-marked rickets, were kept till nineteen weeks old. The former had the same allowance of whole milk, the latter had 250 c.c. of whole milk with 10 grm. of butter per day. When killed the rachitic condition was found in both cases to have advanced. The added butter-fat had produced no curative effect. (Plate II, figs. 7 and 8.)

Figs. 2, 4 and 6 (Plate I) show the condition of the bones as indicated by X-ray photographs in illustrative cases, and Figs. 1, 3 and 5 the appearance of some of the pups at the end of the experiment.

Photographs of the bones by X-ray and of all the pups at the end of the experiment are preserved in the laboratory and may be seen.

Table I (p. 86) gives the details of the experiments.



CHARTS 1 AND 2.—Showing energy value of diets in Calories per kilo. of body-weight throughout experiments on Litters V and VI. Litter V, D 3, very slight rickets. B 1, slight rickets. B 2, rickets. Litter VI, B 5 and D 1, slight rickets. B 6, D 4, D 3 and D 2, rickets.

Such a series of observations shows that:

(1) A diet containing 250 c.c. of whole milk—about 125 c.c. per kilo.—with no less than from 7·6 to 3 grm. of milk-fat per kilo. (see Table I) did not prevent the onset of rickets in pups kept in the laboratory, although they remained otherwise healthy, thus confirming our previous results.

(2) The pups on the separated milk diet and those on the reduced whole-milk diet of 150 c.c. developed rickets sooner and more markedly than those on the full whole-milk diet of 250 c.c. and on the dried milk diet.

Charts 1 and 2 show the energy value per kilo. throughout these experiments, the decrease in each case being due to the gain in weight of the animals. The graphs are simplified by the use of a straight line in place of a curve, the result of the rate of growth modified by the increase of oatmeal and, in some cases, by the reduction in the milk. The more complex curve can be readily constructed from the data given in the table.

It will be noticed that in both experiments the dogs with the lowest energy intake became the most markedly rachitic, with the exception of B 6, a small pup which grew little and which, although the energy intake was as great as that of B 5 and D 1, became distinctly rickety.

It is thus suggested that the development of rickets in pups fed upon oatmeal and separated milk, and the less rapid development upon oatmeal and whole milk is due not to the absence of any anti-rachitic factor but rather to a defective supply of energy in relationship to the rate of growth. A diet which contained sufficient energy for the slow-growing pup might be deficient for a more rapidly growing animal, and this might explain Mellanby's contention that rapidly growing animals are more susceptible to rickets.

In his experiments the effect of a diet of bread with skimmed milk is compared with that of one with bread and whole milk, the bread in both cases being given *ad libitum*. But it is difficult to get pups to eat more than a certain proportion of bread to milk, and hence the energy intake of the former diet must have been considerably less than the latter. While whole milk yields about 60 Calories per 100 grm., separated milk yields only about 35 Calories.

It is not suggested that a low energy value of the diet is the cause of rickets. The first and second experiments recorded by us show that pups on an ample diet may develop the disease. All that is suggested by this experiment is that a sufficient energy intake may decrease the susceptibility to the disease apart from the influence of any accessory factor, and this seemed to us to require further investigation.

Hess and Unger [5] have recorded observations on infants which suggest that the energy value of the food, in whatever proximate principle supplied, may have an important influence.

Litters VII, VIII and IX.

In order to test whether the onset of rickets may be retarded by giving an ample supply of energy apart from any accessory factor associated with milk-fat, the following experiment was arranged and carried out.

To secure that the diet should be uniform, dried milk (Cow and Gate brand) was used in place of sweet milk except in the case of three pups one of which died. The previous experiment had shown that dried milk may safely be substituted for sweet milk.

The samples of dried milk purchased were analysed and the energy value directly determined in the calorimeter.

	Fresh cow's milk.	Cow and Gate dried milk.	
		Full cream.	Separated.
Moisture	—	5·65	8·75
Ash	—	6·25	7·00
Fat	3·0 per cent.	21·00	2·08
Casein	3·5 ,,	27·35	34·36
Lactose	4·8 ,,	39·72	47·81

The bread was purchased in large quantities, dried, analysed and kept in a dry chamber.

Fortunately, three separate litters of pups, in all seventeen animals, were

available at the same time, so that the effects of the treatment could be tested on different strains—a point of no little importance.

The plan of the experiment was to put groups of pups from the different litters, so arranged that some of each litter should be in each group, upon the different diets to be tested.

Litter.	No. in litter.	Born.	Put on diet.	Killed.	Age in weeks.
VII	8	15 : 3 : 20	19 : 4 : 20	13 : 7 : 20 (*D 1 and D 2 not killed*)	17
VIII	6	17 : 3 : 20	19 : 4 : 20	13 : 7 : 20 (*B 6 not killed*)	17
IX	3 (1 died, 23 : 6 : 20.)	21 : 3 : 20	21 : 4 : 20	5 : 7 : 20 (These died under anæsthetic)	15

In two sets of pups the diet contained an abundance of milk-fat, (*a*) in one in fresh milk, and (*b*) in the other in dried full cream milk (Cow and Gate). In the other sets the milk-fat was reduced to a mimimum by the substitution of separated dried milk (Cow and Gate) for whole milk, the energy thus withdrawn being made up by (*a*) in the one set, the addition of lard, a fat which the Vitamine Committee classify as devoid of their hypothetical anti-rachitic substance ([3], p. 83), and (*b*) in the other set, an increase in the amount of bread and of separated milk.

The diets were:

A. Whole fresh milk and bread.

B. Whole dried milk (Cow and Gate) and bread; the bread was the same in amount as in A, and the milk-powder in an amount yielding equal energy value with the whole milk.

C. Separated milk (Cow and Gate) of the same amount as in B, and with the deficient fat supplied by 10 grm. of lard, with the same amount of bread.

D. Separated milk (Cow and Gate) with bread.

The intention was to make up the energy value in group D to the same as the other groups by increasing the bread ration, but it was found impossible to get the pups to eat this, and the energy had to be supplied by increasing the separated milk to yield the energy required.

At the beginning of the experiment, when the pups were five weeks old, the diets were:

A. Cow's milk (fresh)	170 c.c.
Bread	30 grm.
B. Full cream dried milk (Cow and Gate)	22·5 grm.
Water	150 c.c.
Bread	30 grm.
C. Separated dried milk (Cow and Gate)	22·5 grm.
Water	150 c.c.
Bread	30 grm.
Lard	4 grm.
D. Separated dried milk (Cow and Gate)	22·5 grm.
Water	150 c.c.
Bread	40 grm.

This is equivalent to about 195 Calories.

As the pups grew the diets were increased so that the energy supplied per kilo. of pup remained as nearly as possible the same. The adjustment proved somewhat difficult, as is shown in the following table.

Energy Value of Diets per Kilo. at Different Ages.

Diet. Age in weeks	5.	8.	11.	14.	At end of experiment
A. Sweet milk	190	248	256	237	237
B. Full cream	178	221	246	163	138
C. Separated and lard	186	218	242	212	169
D. Separated and bread	211	257	211	192	167
Average	191	236	239	202	177

At the age of eight weeks the diet was increased to the following amounts, on which the pups were kept till the age of eleven weeks:

A. Cow's milk (fresh)	255 c.c.
Bread	45 grm.
B. Full cream dried milk (Cow and Gate)	34 grm.
Water	225 c.c.
Bread	45 grm.
C. Separated dried milk (Cow and Gate)	34 grm.
Water	225 c.c.
Bread	45 grm.
Lard	6 grm.
D. Separated dried milk	34 grm.
Water	225 c.c.
Bread	60 grm.

This is equivalent to about 240 Calories per kilo.

At eleven weeks of age the diets were raised to those given below:

A. Cow's milk	340 c.c.
Bread	60 grm.
B. Full cream dried milk (Cow and Gate)	45 grm.
Water	225 c.c.
Bread	60 grm.
C. Separated dried milk (Cow and Gate)	45 grm.
Water	225 c.c.
Bread	60 grm.
Lard	8 grm.
D. Separated dried milk (Cow and Gate)	56 grm.
Water	375 c.c.
Bread	60 grm.

This is equivalent to about 213 Calories per kilo.

At fourteen weeks of age the pups were put upon the diets increased to the following amounts:

A. Cow's milk (fresh)	425 c.c.
Bread	75 grm.

B. Full cream dried milk (Cow and Gate) . .	56 grm.
Water	300 c.c.
Bread	75 grm.
C. Separated dried milk (Cow and Gate) . .	56 grm.
Water	300 c.c.
Bread	75 grm.
Lard	10 grm.
D. Separated dried milk	70 grm.
Water	375 c.c.
Bread	75 grm.

This is equivalent to about 205 Calories per kilo., and at the end to 180 Calories.

The energy intake was thus initially smaller, but later larger than in the previous Litters V and VI, and this is associated with more rapid growth.

In the separated milk diet the milk-fat per kilo. was initially smaller, and at the end not much greater than in Litters V and VI. *A substance associated with milk-fat did not appear to influence the rate of growth.*

Two pups on the separated milk and bread diet were kept in a large kennel with an open yard, and were freely exercised every day.

The risk of fæcal infection and the influence of lice or fleas were minimised by very special precautions as to cleanliness. One of the laboratory attendants devoted his whole time to the care of the animals, and the work was done most conscientiously. The pups in the laboratory were kept in a large, sunny room upon a concrete floor, enclosed in pens made of rabbit netting. Each pen measured 5 ft. 6 in. square. The pens were moved every day. The pups were fed separately—in groups—and weighed every week. In order to try to hasten the onset of rickets, two upon the diet of separated milk and bread were kept in a rabbit-hutch from twelve weeks of age to sixteen weeks of age. The man in charge found it extremely difficult to keep these two clean and free from vermin, but this was done by frequently changing the hutch, and the pups remained perfectly well.

Those on fresh milk, on account of their small average gain in weight, show a high energy intake to the end of the experiment. Those on dried full cream milk, on account of their marked gain in weight, show a distinct decrease of the energy intake, while those upon separated dried milk and lard, and those upon separated dried milk and bread, show a less decrease on account of their lesser increase in weight.

The experimental results are given in Tables II–VI (pp. 87 to 91), and the rate of growth and energy value of the diets in Charts 3–8 (pp. 91 to 94).

At the end of the experiments, when the pups of Lots VII and VIII were eighteen weeks of age and those of Lot IX fifteen weeks old, each had the two fore-legs X-rayed. They were then killed and the bones of one fore-leg were sent to Prof. Hopkins, while those of the other were used for examination of the epiphyseal cartilages.

Only one of these sixteen pups—VII, D 3—which had begun to lose weight before it was killed, developed rickets and the condition was not diagnosed till the X-ray photograph was examined. Figures of the condition of the right wrist in this and in another pup of the same litter and group (B 5)

are given (Figs. 10 and 11, Plate II). The photographs of the others are not reproduced, but they are preserved in the laboratory and may be inspected.

These results do not seem capable of explanation on the theory that rickets is caused by the absence from the diet of an anti-rachitic factor associated with milk-fat.

It might be urged that the separated milk may contain an amount of an anti-rachitic factor sufficient to prevent the onset of the disease. But the basis of Mellanby's experimental observations is the finding that separated milk up to 250 to 350 c.c. and white bread even with linseed oil, yeast and orange juice will not prevent the onset of rickets in pups between six weeks and twelve weeks of age, while whole milk will prevent it (2), and upon this is based the practical recommendation of the Vitamine Committee that full-cream milk should be used in children for the prevention of the disease (3, p. 99).

Butter is placed with cod-liver oil (3, p. 102) as the substance richest in "fat-soluble A or anti-rachitic factor," and yet in our Experiments I and II butter failed to prevent the onset of rickets and it failed to cure it in a pup of Litter VI. Spontaneous recovery from rickets occurs in dogs as in children, and the recorded cases of "cure" by the administration of butter fat may have been due to this.

Our previous experience has taught us how difficult it is to rear pups free of rickets in a laboratory, in spite of the most generous diets. The results of a large series of observations recorded by Renton and M. Robertson (6) is a striking illustration of this.

The outbreak of rickets in a kennel of fox-hounds at Adelaide, recorded by Bull (7), in which removal of some of the pups to fresh ground prevented the onset of the disease, suggests very strongly that some bacterial infection plays a part in the development of rickets, possibly of the same non-specific character as that which the investigations of McCarrison have shown to be the causal factor in goitre. Morpurgo's (8) results upon young white rats also point in the same direction.

The experiment recorded in the *British Medical Journal* in 1908 (4) shows that pups kept in the country on a fat-poor diet may escape rickets, while those of the same litter, segregated in a laboratory on a fat-rich diet, may develop it.

The first experiment of the present series definitely confirms the experience that under usual laboratory conditions an abundant supply of milk-fat may fail to prevent the onset of rickets and that the addition of butter-fat to the diet has no curative action. It shows that dried milk may be substituted for fresh milk with safety in the diet of pups.

It suggests that a low energy intake may predispose to the development of the disease.

The last experiment shows that by scrupulous care as to cleanliness pups may sometimes be reared in a laboratory without developing rickets, on a diet of separated dried milk and bread, if an ample supply of energy—not necessarily in fats supposed to contain an anti-rachitic factor—is afforded.

The records of growth show that the rate is not strictly proportionate to the energy value of the diet, but that it is modified by other factors (Table VI).

The breed of the dog seems to play a part. The size of the fathers of the different litters is not known, but the pups of Litter VII, from a large, heavy collie, gained on an average 235 grm. per week, those of Litter VIII, from a smaller sheep collie, 170 grm. per week, and those of Litter IX, from a large, old collie, 146 grm. per week.

Our records also indicate that *pups kept in the open air grow more rapidly than those confined in the laboratory*. Chart 9 (p. 94), which gives the rate of growth of the pups used in Experiment I (1) and a comparison of Chart 8 with Charts 6 and 7 show this very clearly.

CONCLUSIONS.

(1) In young dogs under ordinary laboratory conditions a liberal allowance of milk-fat, up to even 14 grm. per kilo. of body-weight, neither prevents the onset of rickets nor cures it when it has developed.

(2) Pups kept largely in the open air may escape the development of rickets on an intake of less than 1 grm. of milk-fat per kilo. of body-weight.

(3) With scrupulous care as to cleanliness it is, at least sometimes, possible to rear pups free of rickets in the laboratory on an intake of only about 0·5 grm. of milk-fat per kilo. of body-weight, along with bread, provided that the diet affords an adequate supply of energy.

(4) The energy value of the diet, however supplied, quite apart from the presence of any hypothetical anti-rachitic factor in milk-fat, would seem to play a part in controlling the development of rickets, but that it is only a contributory part is shown by the development of rickets in pups with a high energy intake if confined in the laboratory without scrupulous care as to cleanliness.

(5) Milk-fat may be reduced to about 0·3 grm. per kilo. of body-weight, if its place is taken by an equal amount of lard, without the onset of rickets.

(6) The results of these obervations do not support the conclusion of the Accessory Food Factors Committee of the Medical Research Council that rickets is a deficiency disease due to a lack of an anti-rachitic factor associated with milk-fat.

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FIG. 8.

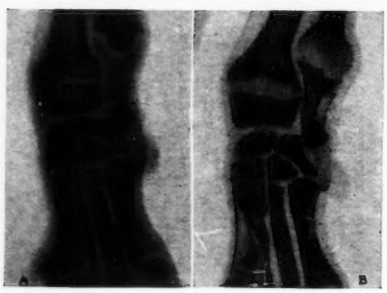

FIG. 7.

FIG. 9.

FIG. 10. FIG. 11.

Plate II. Noël Paton and Watson. Rickets.

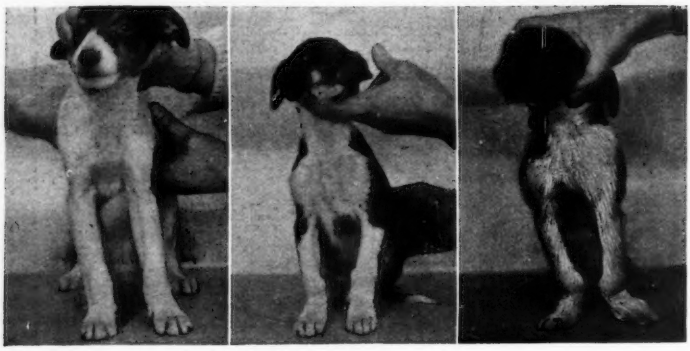

FIG. 1. FIG. 3. FIG. 5.

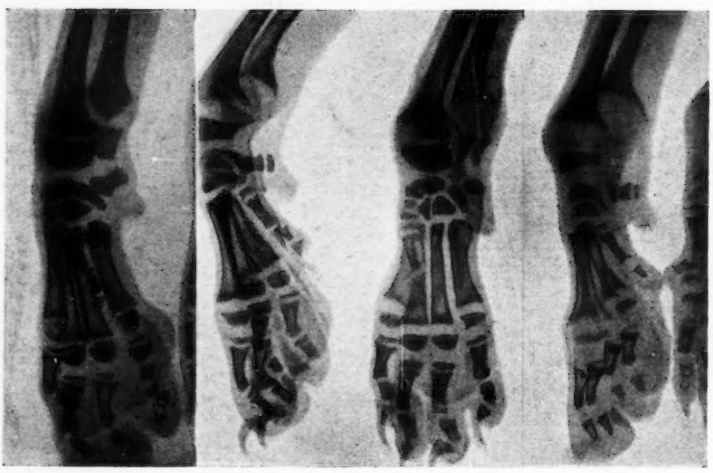

FIG. 2. FIG. 4. FIG. 6.

Plate I. Noël Paton and Watson. Rickets.

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EXPLANATION OF PLATES.

PLATE I.

Figs. 1 and 2.—Litter V, Dog 3, fed on oatmeal porridge and dried full-cream milk = 11 grm. milk fat. Energy value of diet 377 C. at start, 437 C. at end.

Figs. 3 and 4.—Litter V, Bitch 1, fed on oatmeal porridge and fresh whole milk = 7·5 grm. milk fat. Energy value of diet 270 C. at start, 330 C. at end.

Figs. 5 and 6.—Litter V, Bitch 2, fed on oatmeal porridge and separated milk, 0·8 grm. milk fat. Energy value 207 C. at start, 247 C. at end.

PLATE II.

Fig. 7.—Litter VI, Dog 1: A. Skiagram on 11 : 2 : 20 after being kept on porridge and 250 c.c. whole milk. B. Skiagram on 10 : 3 : 20 after another month on this diet. Note marked advance in the rachitic condition.

Fig. 8.—Litter VI, Dog 4.: A. Skiagram on 11 : 2 : 20 after being kept upon porridge and 150 c.c. separated milk. B. Skiagram on 10 : 3 : 20 after being kept for a month upon porridge with 250 c.c. whole milk and 10 grm. butter = 15·5 grm. milk-fat. Note advance in rachitic condition.

Fig. 9.—Litter VI: A. Bitch 5 and B. Dog 2, both on oatmeal porridge, but the former with 250 c.c. whole milk = 7·5 grm. milk-fat, energy value 270 C. at start, 330 C. at end; the latter with 250 c.c. separated milk = 0·8 grm. milk-fat, energy value 207 C. at start, 217 C. at end. Note much more marked rickets in the second.

Fig. 10.—Litter VII, Dog 3: Kept under the same conditions as Bitch 5. The only pup of nine from three litters which developed rickets on this diet.

Fig. 11.—Litter VIII, Bitch 5: Skiagram after being kept on bread and dried separated milk = 0·48 grm. milk-fat, energy value 364 C. at start, 549 C. at end. Note absence of rickets in this as in 8 other pups on the same diet.

TABLE I.

No.	Diet.	Milk-fat per day. Total.	Milk-fat per day. Per kilo. First.	Milk-fat per day. Per kilo. Last.	Energy value in calories. For first 4 weeks.	Energy value in calories. For last 6 weeks.	Weight in grm. Initial.	Weight in grm. Terminal.	Increase in weight. Per cent. of initial.	Increase in weight. Average per week from 10 to 20 weeks.	Condition when killed.
					LITTER V.						
	(Each had 30 grm. oatmeal for 5 weeks, afterwards 45 grm.)										
B 1	+ 250 c.c. whole milk (fresh)	7·5	6·5	3·9	270	330	1150	1900	65·2	75	Rickets (condition between B 2 and D 3).
B 2	+ 250 c.c. separated milk	0·8	0·6	0·4	207	247	1300	2000	53·8	70	Marked rickets.
D 3	+ 30 grm. dried whole milk	11·0	9·0	4·1	377	437	1150	2700	134·7	155	Slight rickets.
					LITTER VI.						
					Whole milk.					(From 5 to 15 weeks.)	
B 5	+ 250 c.c. whole milk	7·5	7·6	2·9	270	330	990	2575	160·1	158·5	Slight rickets.
D 1	+ „ „ „	7·5	7·3	2·7	270	330	1020	2770	171·5	175	Slight rickets. (Kept alive.)
B 6	+ „ „ „ then 150 c.c.	7·5 then 4·5	8·5	2·2	270	270	880	2070	135·2	119	Rickets.
					Separated milk.						
D 2	+ 250 c.c. separated milk	0·8	0·72	0·32	207	267	1100	2450	140·7	135	Rickets.
D 3	+ 250 c.c. separated milk, then 150 c.c.	0·8 then 0·48	0·74	0·22	207	232	1080	2121	96·3	104	Rickets.
D 4	+ 250 c.c. separated milk, then 150 c.c.	0·8 then 0·48	0·87	0·22	207	232	920	1680	82·6	76	Rickets. (Kept alive. Sweet milk and butter.)

TABLE II.—*Litters VII, VIII and IX (5th to 8th week of age).*

No.	Diet.	Milk-fat per day.			Energy value in calories.			Body-weight in grm.		
		Total.	Per kilo. Initial.	Per kilo. Terminal.	Total.	Per kilo. Initial.	Per kilo. Terminal.	Initial.	Terminal.	Gain per cent.
Lot VII, B 8	A.—Whole milk 170 c.c.; bread 30 grm.	4·6	3·83	3·17	215·26	179·4	148·5	1200	1450	20·8
Lot VIII, B 6			4·28	3·91		200·2	183·2	1075	1175	9·3
Lot VII, D 2	B.—Full cream milk (dried) 22·5 grm.; bread 30 grm.	4·5	3·0	2·1	221·71	138·5	105·5	1600	2100	31·0
„ D 5			3·5	2·7		173·8	134·3	1275	1650	29·0
Lot IX, D 1			4·5	3·8		221·7	188·6	1000	1175	17·5
Lot VII, D 1	C.—Separated milk (dried) 22·5 grm.; bread 30 grm.; lard 4 grm.	0·45	0·26	0·2	237·0	139·4	110·2	1700	2150	26·0
„ D 4			0·33	0·27		178·8	143·6	1325	1650	24·0
Lot VIII, D 2			0·4	0·31		204·7	163·4	1125	1450	28·9
„ B 4			0·4	0·4		215·4	215·4	1100	1100	0·0
Lot VII, D 3	D.—Separated milk (dried) 22·5 grm.; bread 40 grm.	0·45	0·33	0·28	243·0	180·0	154·2	1350	1575	16·0
„ B 7			0·4	0·32		221·0	174·8	1100	1390	26·0
Lot VIII, D 3			0·4	0·32		216·0	174·8	1125	1390	23·0
„ B 5			0·4	0·34		216·0	183·8	1125	1300	15·0
Lot IX, D 2			0·36	0·28		190·6	154·3	1275	1575	23·5
„ D 3			0·39	0·31		211·3	160·6	1150	1450	26·0
„ D 4			0·33	0·26		180·0	143·0	1350	1700	25·9
„ B 5			0·4	0·32		226·0	176·7	1075	1375	27·0
„ B 6			0·38	0·33		207·0	180·0	1175	1350	14·8

TABLE III (*8th to 11th week of age*).

No.	Diet.	Milk-fat per day. Total.	Milk-fat per day. Per kilo. Initial.	Milk-fat per day. Per kilo. Terminal.	Energy value in calories. Total.	Energy value in calories. Per kilo. Initial.	Energy value in calories. Per kilo. Terminal.	Body-weight in grm. Initial.	Body-weight in grm. Terminal.	Body-weight in grm. Gain per cent.
Lot VII, B 8	A.—Whole milk 225 c.c.; bread 45 grm.	7·2	4·96	4·11	322·89	223·0	184·0	1450	1750	20·6
Lot VIII, B 6			6·11	4·7		274·0	211·0	1180	1530	30·0
Lot VII, D 2	B.—Full cream milk (dried) 34 grm.; bread 45 grm.	6·75	3·21	2·33	343·0	163·3	118·0	2100	2900	38·0
,, D 5			4·09	3·0		208·0	152·0	1650	2250	36·3
Lot IX, D 1			5·77	5·2		292·0	264·0	1175	1300	11·1
Lot VII, D 1	C.—Separated milk (dried) 34 grm.; bread 45 grm.; lard 6 grm.	0·675	0·31	0·22	355·45	165·0	118·0	2150	3000	39·3
,, D 4			0·41	0·26		215·0	139·0	1650	2550	54·5
Lot VIII, D 2			0·47	0·35		245·0	182·0	1450	1950	34·5
,, B 4			0·61	0·5		323·0	263·0	1100	1350	22·7
Lot VII, D 3	D.—Separated milk (dried) 34 grm.; bread 60 grm.	0·675	0·43	0·28	364·45	231·0	152·0	1580	2400	51·9
,, B 7			0·48	0·26		260·0	143·0	1400	2550	82·1
Lot VIII, D 3			0·48	0·29		260·0	158·0	1400	2300	64·3
,, B 5			0·52	0·39		280·0	208·0	1300	1750	34·6
Lot IX, D 2			0·43	0·3		231·4	167·2	1575	2180	38·4
,, D 3			0·47	0·35		251·0	192·0	1450	1900	31·0
,, D 4			0·39	0·3		214·3	165·6	1700	2200	29·4
,, B 5			0·49	0·39		264·0	208·0	—	1750	—
,, B 6			0·5	0·36		269·9	194·0	1350	1875	38·8

TABLE IV (*11th to 14th week of age*).

No.	Diet.	Milk-fat per day. Total.	Milk-fat per day. Per kilo. Initial.	Milk-fat per day. Per kilo. Terminal.	Energy value in calories. Total.	Energy value in calories. Per kilo. Initial.	Energy value in calories. Per kilo. Terminal.	Body-weight in grm. Initial.	Body-weight in grm. Terminal.	Body-weight in grm. Gain per cent.
Lot VII, B 8	A.—Whole milk 340 c.c.; bread 60 grm.	9·2	5·25	4·2	430·5	246·0	195·7	1750	2200	25·7
Lot VIII, B 6			6·03	3·9		282·3	183·2	1525	2350	54·0
Lot VII, D 2	B.—Full cream milk (dried) 45 grm.; bread 60 grm.	9·0	3·1	2·1	443·4	152·9	106·8	2900	4150	42·0
„ D 5			4·0	3·0		197·0	147·8	2250	3000	30·0
Lot IX, D 1			6·9	6·0		141·0	295·6	1300	1500	15·3
Lot VII, D 1	C.—Separated milk (dried) 45 grm.; bread 60 grm.; lard 8 grm.	0·9	0·45	0·25	473·9	130·0	131·6	2000	3600	20·0
„ D 4			0·35	0·23		185·8	118·5	2550	4000	56·8
Lot VIII, D 2			0·46	0·34		243·0	186·1	1950	2600	33·8
„ B 4			0·67	0·47		351·0	249·4	1350	1900	41·0
Lot VII, D 3	D.—Separated milk (dried) 56 grm.; bread 60 grm.	1·12	0·47	0·34	443·5	148·8	134·4	2400	3300	37·5
„ B 7			0·44	0·30		173·9	119·8	2550	3700	45·0
Lot VIII, D 3			0·49	0·37		193·6	147·3	2290	3000	31·0
„ B 5			0·64	0·41		253·4	164·2	1750	2700	54·2
Lot IX, D 2			—	—		—	—	2180	—	—
„ D 3			0·59	0·51		233·4	197·1	1900	2250	18·4
„ D 4			0·51	0·39		201·5	155·6	2200	2850	29·5
„ B 5			0·73	0·47		253·4	201·5	1750	2200	25·7
„ B 6			0·60	0·43		236·5	169·2	1875	2620	39·7

TABLE V (*after* 14 *weeks of age*).

No.	Diet.	Milk-fat per day. Total.	Milk-fat per day. Per kilo. Initial.	Milk-fat per day. Per kilo. Terminal.	Energy value in calories. Total.	Energy value in calories. Per kilo. Initial.	Energy value in calories. Per kilo. Terminal.	Body-weight in grm. Initial.	Body-weight in grm. Terminal.	Gain per cent.
Lot VII, B 8	A.—Whole milk 425 c.c.; bread 75 grm.	12·75	5·80	5·10	562·6	256	225	2200	2500	13·6
Lot VIII, B 6			5·42	4·55		239	201	2350	2800	19·1
Lot VII, D 2	B.—Full cream milk (dried) 56 grm.; bread 75 grm.	11·25	2·71	2·37	571·7	138	120	4150	4750	14·5
,, D 5			3·75	3·21		190·5	163	3000	3500	16·0
Lot IX. D 1			—	—		—	—	—	—	—
Lot VII, D 1	C.—Separated milk (dried) 56 grm.; bread 75 grm.; lard 10 grm.	1·12	0·31	0·27	592·4	165·5	143	3600	4150	15·3
,, D 4			0·28	0·24		148	128	4000	4625	15·6
Lot VIII, D 2			0·43	0·37		228	197	2600	3000	15·4
,, B 4			0·59	0·47		312	249	1900	2375	25·0
Lot VII, D 3	D.—Separated milk (dried) 70 grm.; bread 75 grm.	1·4	0·42	0·37	549·1	167	144·5	3300	3800	15·0
,, B 7			0·38	0·31		148	123	3700	4460	20 5
Lot VIII, D 3			0·47	0·37		183	144·5	3000	3800	26·6
,, B 5			0·52	0·41		203	163	2700	3375	25·0
Lot IX, D 2*			—	—		—	—	—	—	—
,, D 3			0 62	0·74		244	289	2250	1900	−15·5
,, D 4			0·49	0·43		192·6	170·2	2850	3225	13·0
,, B 5			0·64	0·56		250	220	2200	2500	13·6
,, B 6			0·53	0·48		209·6	190·6	2620	2880	10·0

* Died.

TABLE VI.—*Average gain per week on diets—*

A.	Lot VII,	B 8	118·0 grm.,	11 weeks.
	Lot VIII,	B 6	144·0 ,,	12 ,,
B.	Lot VII,	D 2	286·0 ,,	11 ,,
	Lot VII,	D 5	202·0 ,,	11 ,,
	Lot IX,	D 1	55·5 ,,	died at end of 9th week of diet.
C.	Lot VII,	D 1	222·7 ,,	11 weeks.
	,,	D 4	300·0 ,,	11 ,,
	Lot VIII,	D 2	156·0 ,,	12 ,,
	,,	B 4	106·0 ,,	12 ,,
D.	Lot VII,	D 3	222·7 ,,	11 ,,
	,,	B 7	305·0 ,,	11 ,,
	Lot VIII,	D 3	222·9 ,,	12 ,,
	,,	B 5	187·5 ,,	12 ,,
	Lot IX,	D 2	142·8 ,,	11 ,,
	,,	D 3	68·0 ,,	11 weeks; going down in weight before death.
	,,	D 4	158·0 ,,	11 weeks.
	,,	B 5	129·5 ,,	11 ,,
	,,	B 6	155·0 ,,	11 ,,

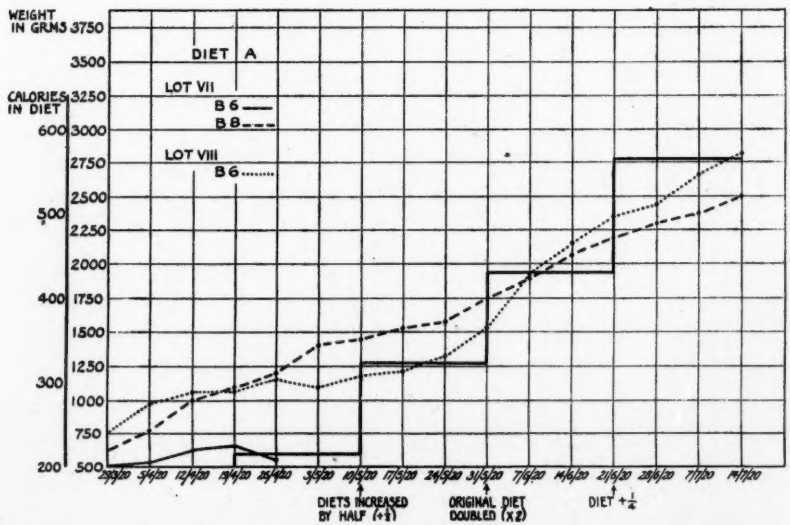

CHART 3.—Growth curves and energy value of diet of pups on fresh whole milk and bread.

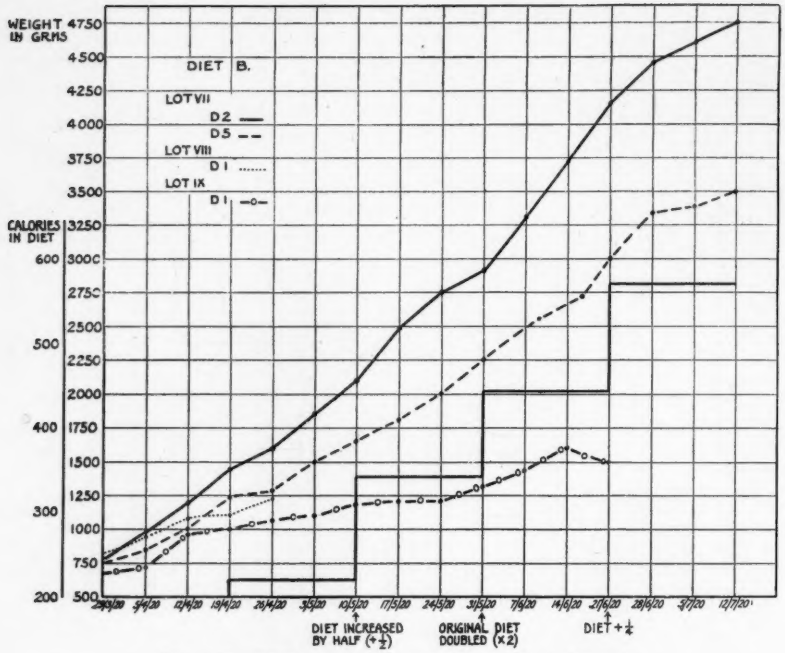

CHART 4.—Ditto on whole cream, dried milk and bread.

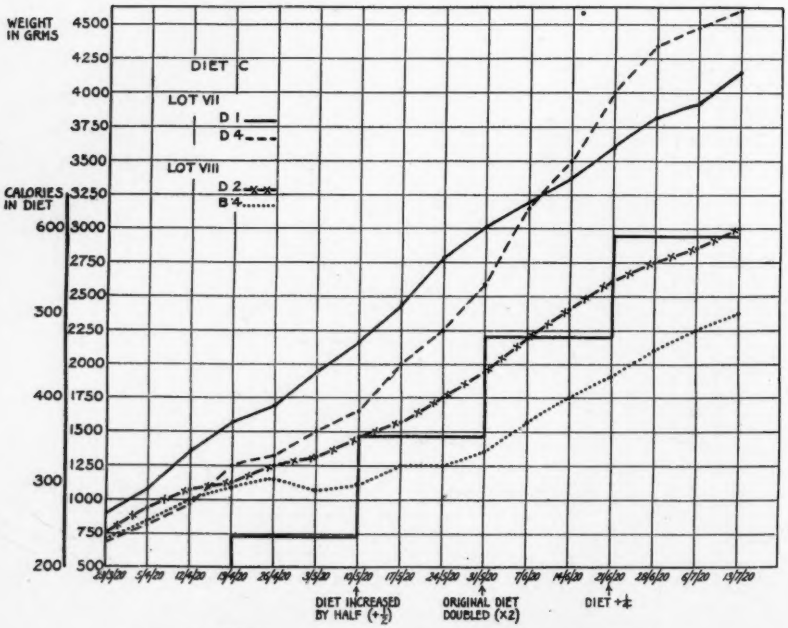

CHART 5.—Ditto on separated dried milk and bread with lard.

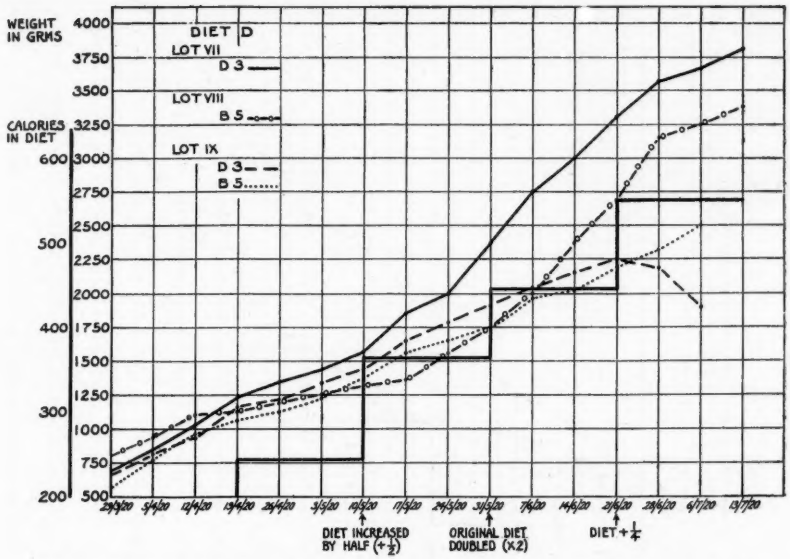

CHART 6.—Ditto on separated milk and bread, the pups being kept in a large pen in the laboratory.

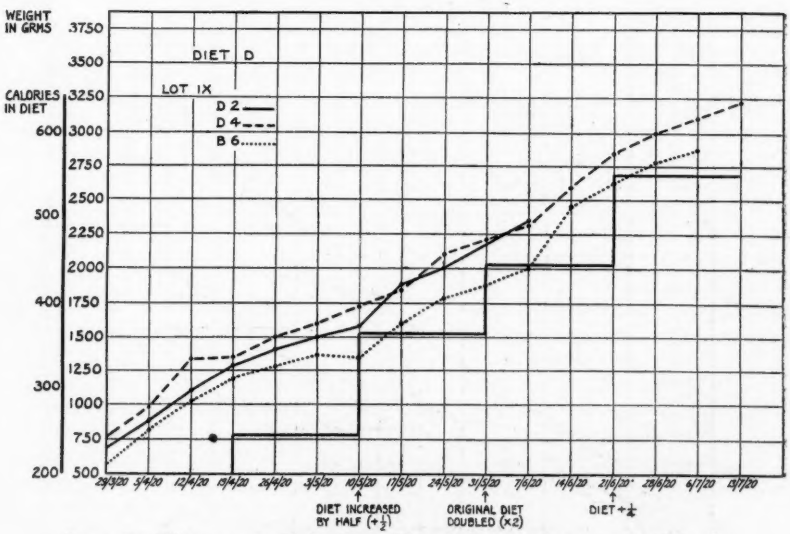

CHART 7.—Ditto on separated milk and bread, the pups being confined for the last three weeks of life.

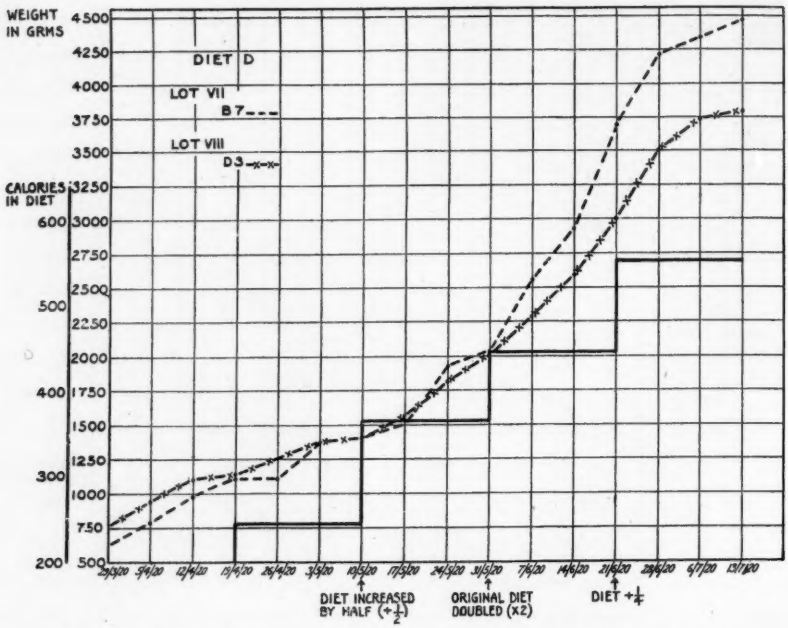

CHART 8.—Ditto on separated dried milk and bread, the pups being kept in the open air. Compare with D 3 and B 5 in Chart 6.

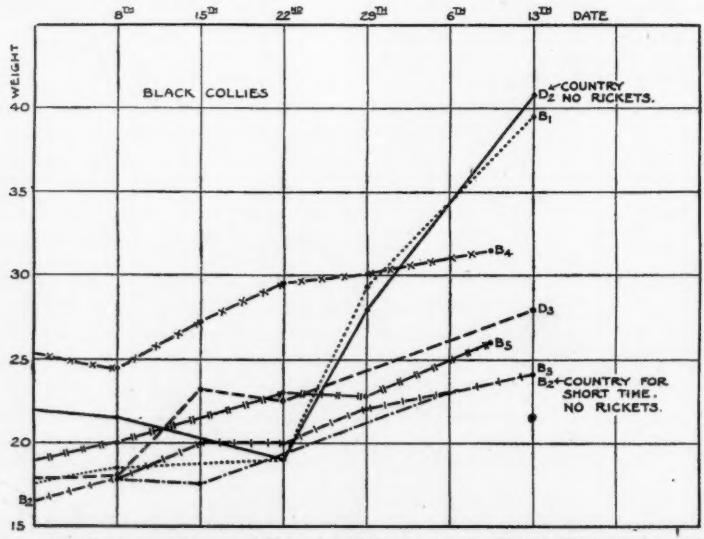

CHART 9.—Growth curves of pups of Experiment I recorded in *British Medical Journal* (1). D 2 and B 1 kept in the open air. B 2 kept in the open air for two weeks and then returned to the laboratory. None of these developed rickets. The others kept in the laboratory all developed rickets.

THE ANTISEPTIC POTENCY OF ACRIFLAVINE, WITH CONSIDERATIONS ON THE VARIABILITY OF RESULTS IN TESTING ANTISEPTICS.

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THE method very commonly adopted in defining the value of an antiseptic is to express its potency as the ratio of the reciprocals of the sterilising concentrations of the substance in question and some standard compound, commonly phenol. This procedure probably owes its acceptance in considerable part to the fact that it appears to eliminate irregularities due to variations, which are observed when the sterilising concentration of any single substance is determined on repeated occasions. In the case of the ratio, it is assumed that alterations in the resistance of the test organism, whether due to differences in the nutrient medium or to other causes, will affect the results with different antiseptics comparably and in the same direction, so that repeated experiments with the same pair of substances will yield a constant value. A method of this kind does not take into account the fact that alterations in hydrogen-ion concentration of the solution may affect the potency of different antiseptics in opposite directions, *e. g.* the action of mercuric chloride is enhanced when the reaction passes to the acid side, whereas the flavine compounds act best in more alkaline medium, as we have shown (3). A further circumstance which may interfere with the utility of the ratio as an expression of the value of an antiseptic, is that it is commonly calculated from experiments in which the period of contact of the organisms with the antiseptic agent is limited to a few minutes. Certain substances, *e. g.* phenol and mercuric chloride, act rapidly. On the other hand, there are many powerfully antiseptic organic compounds, such as those of the diamino-acridene group ("flavines"), which cause death of the organisms comparatively slowly; but, short of lethal action, they exert a powerful inhibitory or "bacteriostatic" effect, which can be advantageously utilised in therapy, *e. g.* in the case of infected wounds (1, 2).

When the preparation of the flavine compounds was first commenced on a commercial scale, it appeared to us advisable to make a careful comparison of

the biological properties of different specimens, since organic chemical syntheses under conditions such as are involved in the production of these substances might give rise to mixtures including the products of side-reactions, whose behaviour on the tissues and on bacteria had not been investigated. The results showed that, as regards antiseptic power, a series of about forty commercial products did not differ from each other notably even when some impurity, in the form of insoluble matter, was present in varying degree. On the other hand, marked differences were found in irritating properties, as tested on the conjunctiva; this is probably a point of some considerable importance when the antiseptic is to be brought into contact with a very sensitive mucous membrane, such as that of the urethra. These systematic tests led to the accumulation of an extensive series of data regarding the absolute concentrations of diaminoacridene compounds which serve to inhibit growth and to sterilise inoculated media. The same compounds have been tested repeatedly and also different specimens have been compared. The results are now detailed in the case of those specimens of diaminoacridene methochloride—"acriflavine"—which have been most frequently examined, as they illustrate the range of variation which may be expected in a series of tests of this kind.

The tests were carried out in serum and in dilute peptone water. Serum was employed as it represents the fluid constituent to which antiseptics in contact with the tissues are exposed; also, it is to be regarded as extremely constant in composition and reaction, which are important properties in a medium employed in testing antiseptic action. The peptone water solution consisted of 0·7 per cent. bacteriological peptone along with 0·35 per cent. sodium chloride. At first the important effect of small alterations in the hydrogen-ion concentration of this solution was not appreciated and the earlier tests were made on a medium with a P_H value below 6; subsequently a solution with a P_H value of 7·2 to 7·8, approximately, was employed. The striking increase of antiseptic potency in the more alkaline medium is well shown in Tables III and IV. The experiments with *B. coli* will be considered most fully, as there tended to be greater variation with this organism than with staphylococcus.

The procedure adopted was as follows: Ox serum was employed; the blood was obtained at the slaughter-house, and was received directly from the severed neck-vessels into a sterile bottle in which it was allowed to clot; the serum was pipetted off 24 to 48 hours later and was freed from red corpuscles by centrifuging; then it was heated for about an hour at 56° C. If the serum proved to be sterile it was measured in amounts of 1 c.c. into $3 \times \frac{1}{2}$ in. sterile test-tubes, which were plugged with cotton-wool; these were heated again at 56° C. for half an hour. In conducting a test varying dilutions of antiseptic in watery solution were added, the largest volume not exceeding 0·1 c.c.; then of a 24 hours' culture of the test organism in peptone water diluted with 0·85 per cent. saline 0·1 c.c. was added, the dilution usually employed being 1 : 1000, but the effect with other quantities of bacteria was also tested.

In general the following concentrations of antiseptic were employed: 1 : 1,000,000, 1 : 400,000, 1 : 200,000, 1 : 100,000, 1 : 40,000. The same

strain of *B. coli* was used throughout; it was found that with different specimens of ox serum the amount of growth, as shown by the development of turbidity in the medium, varied considerably; hence inhibition of growth as judged by the eye was likely to be a less satisfactory criterion than sterility as determined by subculturing a loopful of the mixture on agar. The

TABLE I.—*Showing the Sterilising Concentration of Acriflavine for* B. coli *in Ox Serum (56° C.), as Determined in a Series of Sixty-eight Tests.*

Sterilising concentration.	Series A. Products.				Totals.	Series B. Products.		Totals.	Series C. Products.		Totals.	Sum of totals.
	O.	P.	Q.	R.		O.	P.		O.	P.		
1 : 1,000,000	1	—	—	4	**5**	—	2	**2**	—	1	**1**	**8**
1 : 400,000	—	1	1	7	**9**	—	1	**1**	—	7	**7**	**17**
1 : 200,000	1	—	—	7	**8**	4	—	**4**	1	3	**4**	**16**
1 : 100,000	4	—	3	5	**12**	3	—	**3**	8	1	**9**	**24**
1 : 40,000	—	—	2	—	**2**	—	—	—	—	—	—	**2**
< 1 : 40,000*	—	—	1	—	**1**	—	—	—	—	—	—	**1**
No. of tests . . .					**37**			**10**			**21**	**68**

In series A the inoculum = 0·001 c.c. of culture per 1 c.c. of medium; series B = 0·00005 c.c; series C = 0·000025 c.c.

* This indicates that 1 : 40,000 failed to sterilise.

sterilising concentration is therefore taken as the lowest which failed to yield growth in subculture. Table I shows the results obtained in serum with the four specimens of acriflavine which have been most frequently examined.* The modal value, 1 : 100,000, which was obtained in 35 per cent. of the tests, corresponds with that originally assigned to this antiseptic. It is noteworthy

TABLE II.—*Showing the Sterilising Concentration of Acriflavine (Product P) for* B. coli, *as Determined with varying Inoculation Doses on Different Occasions.*

Medium = Ox serum 56° C.

Inoculum per 1 c.c. medium.	Sterilising concentration.
0·1 c.c.	1 : 100,000
0·01 ,, ...	1 : 130,000
0·002 ,, ...	1 : 200,000
0·001 ,, ...	1 : 400,000

that the efficiency of antiseptic action is practically independent of the inoculation doses used, the results with an inoculum of 0·001 c.c. being equal to those with 0·00005–0·000025 c.c. Additional experiments with product "P" on different occasions further emphasise this point (Table II). Thus while the magnitude of the inoculum increased from 1 to 100, the sterilising dose of the

* Product "O" is the original sample of "trypaflavin" given to us by Prof. Ehrlich.

antiseptic only varied from 1 to 4. In view of the general range of variation already noted with the same inoculation dose on various occasions, the orderly arrangement in this table is probably fortuitous. As regards the three experiments in which the sterilising dose was highest (1 : 40,000 or more), it is to be noted that on one of the occasions product " O " was tested simultaneously and the sterilising dose was found to be 1 : 100,000; thus there is no evidence that either the culture or the medium was abnormal on this occasion. The tests have extended over a period of more than four years and it is clear from the records that there is no grouping of the results with reference to time, *i. e.* there is no evidence in the case of *B. coli* that the organism has undergone any permanent or cyclic alteration in susceptibility. When the same product has been tested several times on the same day with the same specimen of serum and culture the results have shown a marked degree of uniformity. Thus product " R " was tested in five series, the only difference being that certain of them were shaken more frequently than others during the first few hours' incubation; the result was that 1 : 1,000,000 sterilised in two series and 1 : 400,000 in three. Again, three specimens of product " P " (one being the specimen as originally furnished and the other two, A and B, prepared from it by recrystallisation) were tested on a single occasion, with the result that in two sterilisation was effected by a concentration of 1 : 400,000 and in the third by 1 : 200,000. As has been noted previously, different specimens of ox serum differ considerably in the luxuriance of growth which they yield when similarly inoculated with *B. coli*, but there appears to be no relationship between this factor and the sterilising concentration of antiseptic.

B. coli in peptone water.—The sterilising dose was originally stated to be 1 : 1300; at that time peptone water with a P_H value of less than 6·0 was employed, as noted above. The great intensification of antiseptic action which results when the P_H value of the medium is 7·2 to 7·8 is well shown in Table III. Thus the sterilising concentration under these conditions is 1 : 20,000.

TABLE III.—*Showing the Effect of Hydrogen-ion Concentration upon the Sterilising Concentration of Acriflavine for* B. coli *in Peptone Water in a Series of Tests.*

Inoculum = 0·001 c.c. culture per 1 c.c. medium.

Sterilising concentration.	P_H of medium. Under 6·0.	7·2–7·8.
1 : 100,000	—	1
1 : 20,000	—	7
1 : 10,000	—	3
1 : 2,000	3	—
1 : 1,300	5	—
> 1 : 1,300*	8	—
1 : 1,000	3	—

*** This indicates that 1 : 1300 failed to sterilise.**

Staphylococcus.—Several strains of *Staphylococcus aureus* were employed at different times, but there was no evidence of any notable difference in their behaviour toward the antiseptic. As is shown in Table IV, the collected results confirm the originally assigned value of 1 : 200,000 as the sterilising concentration in serum (modal value obtained in 51 per cent. of the tests). In the case of peptone water medium, alteration in the hydrogen-ion concentration affects the values, but not to the same extent as occurs with *B. coli.* At P_H 7·2 to 7·8 the sterilising concentration lies in the neighbourhood of 1 : 200,000. It is striking that although serum is a much more suitable medium than peptone water, as shown by the abundance of growth, nevertheless the potency of the antiseptic is on the average identical in the two cases.

TABLE IV.—*Showing the Sterilising Concentration of Acriflavine for* Staphylococcus aureus *as determined in a Series of Tests.*

The inoculum per 1 c.c. of medium in the serum series consisted of 0·001 c.c. in 27 tests, 0·00005 c.c. in 15, 0·000025 c.c. in 19 and 0·000005 c.c. in 3: as no difference is attributable to this factor the results are grouped together. In the peptone water series 0·001 c.c. was used throughout.

Sterilising concentration.	Medium. Ox serum (56° C.). No. of tests.	Peptone water P_H < 6·0. No. of tests.	Peptone water P_H 7·2–7·8. No. of tests.
1 : 400,000	15	1	4
1 : 200,000	32	0	7
1 : 100,000	14	7	0
1 : 40,000	2	8	1
> 1 : 40,000*	0	1	0
Totals	63	17	12

* This indicates that 1 : 40,000 failed to sterilise.

THE ADDITION OF ANTISEPTIC TO THE MEDIUM AFTER GROWTH OF ORGANISMS HAS OCCURRED.

Experiments on the plan of those already described in serum might be considered to involve the fallacy that one is dealing with organisms which are not accommodated to the medium employed, since the inoculum is a peptone water culture. Accordingly the following observation was made: The serum was inoculated first of all, and after incubation for 48 hours at 37° C. varying amounts of acriflavine were added to the resulting growths. The effect was ascertained by making subcultures after a further period of incubation for 48 hours. The result was that the tubes containing the antiseptic in a concentration of 1 : 100,000 and upwards were rendered sterile. Therefore it is a striking fact that a heavy 48-hours' growth of organisms is killed with as great ease by the subsequent addition of the antiseptic as are a few organisms added simultaneously with the antiseptic. The experiment in which organisms are introduced first of all, and then the

antiseptic is added after growth has occurred, resembles more closely than the others the condition met with in treating an infected wound. *B. coli* was employed in these tests.

REPEATED INOCULATION OF MEDIUM CONTAINING THE ANTISEPTIC.

The question as to whether the antiseptic tends readily to undergo deterioration in the serum or to become exhausted by the organisms appears to have been answered in the negative by previous observations ([1, 2]). Thus it was found that bactericidal action progresses for at least 24 to 48 hours at 37° C., and tubes which appear without visible growth at the end of this period do not subsequently show obvious proliferation. The point has been further investigated, however. A series of tubes containing serum with varying amounts of acriflavine were inoculated and kept at 37° C., the presence of viable colon bacilli being tested for by making subcultures on agar with one loopful of each mixture after 24 and 48 hours; at the end of this time they were

TABLE V.—*Showing the Results of Reinoculation Experiments with Acriflavine and* B. coli *in Ox Serum (56° C.).*

Concentration of antiseptic.	Resulting growth from			
	Original inoculation after		Re-inoculation after	
	24 hours.	48 hours.	24 hours.	48 hours.
1 : 1,000,000	+	+	...	...
1 : 400,000	Slight inhibition	Inhibition	...	...
1 : 200,000	3 colonies	—	17 colonies	—
1 : 100,000	3 „	—	25 „	—
1 : 40,000	—	—	—	—

+ = abundant growth in subculture: — = no growth in subculture.

re-inoculated with 0·001 c.c. of *B. coli* culture as usual. The results are shown in Table V. A similar series was re-inoculated after 96 hours; in this case the primary inoculation was sterilised in a concentration of 1 : 100,000, and 24 hours after re-inoculation the same tube yielded twelve colonies, while after 48 hours it was again sterile. Accordingly there is no evidence pointing to the inactivation of the antiseptic in the medium or to exhaustion by the organisms of the minute amounts which are active in producing bactericidal action.

VARIABILITY OF RESULTS.

In seeking for an explanation of the variability in results met with in repeated tests (Tables I and IV), another phenomenon should be taken into account, viz. irregularity in sterilising action which may occur in a series of tubes containing ascending doses of antiseptic. Thus occasionally concentrations of 1 : 130,000 and 1 : 40,000 may sterilise the mixture, while 1 : 100,000 fails. This observation, which is more common with certain other substances of the acridine group, corresponds with that recorded by Richet and Cardot ([6]) on exaggerated variations in the amount of acid formed in different tubes of whey containing a constant concentration of certain antiseptics when

inoculated similarly with a lactic organism. The assumption that a resistant strain of bacteria has been evolved suggests itself in view of the work of Ehrlich and his school on drug-resistance of trypanosomes, and of experiments on the acquired resistance to antiseptics of *V. choleræ* (Shiga [7]) and of the lactic organism (Richet and Cardot [6]). But in the present instance it must be emphasised that if such heightened resistance does in fact exist, it is probably of an inferior grade, and in our experiments, at least, the attempt to produce resistant strains both of *B. coli* and *Staphylococcus aureus* by continued subculture in peptone water medium containing acriflavine has so far been unsuccessful.([1]) Types of coliform bacilli which are highly insusceptible to the flavine compounds have been recovered occasionally from wounds treated with these substances (Drummond and McNee [5]), but the cultural characters of these organisms differ markedly from those of the common types of *B. coli* ([4]), and it appears most probable that their resistance, like that of *B. pyocyaneus*, is natural and not acquired. With regard to the cause of the irregularities we should incline to the following view: In the vicinity of the lethal concentration, especially with substances which destroy the life of the organisms only slowly, there is at first a fairly balanced equilibrium between, on the one hand, the tendency of the organisms to assimilate foodstuffs, and as a result to proliferate, and on the other, the inhibitory action of the antiseptic. Accordingly, it is probable that at this stage some relatively minute difference, which it is at present impossible to define precisely, may disturb the balance in favour of the organisms, thus leading to growth.

CONCLUSIONS.

(1) The sterilising concentrations of acriflavine (diaminoacridene methochloride) in heated ox serum (56° C.) for *B. coli* and *Staphylococcus aureus* are respectively 1 : 100,000 and 1 : 200,000, these being the modal values obtained in an extensive series of tests. These figures correspond with the values originally assigned.

(2) The sterilising concentrations in dilute peptone water, with a reaction P_H 7·2 to 7·8 (approximately), are found to be for *B. coli* 1 : 20,000, and for *Staphylococcus aureus* 1 : 200,000 (modal values). These values represent a much higher activity than those originally given; they are to be explained by the increased efficiency due to using medium of a lower hydrogen-ion concentration than that previously employed.

(3) The sterilising concentration of the antiseptic is within wide limits independent of the size of the inoculum.

(4) Different commercial specimens of acriflavine could not be distinguished as regards their antiseptic potency, but there was evidence that certain specimens were more irritating to the conjunctiva than others; this property is likely to be important where the substance is employed in treating an infection of a delicate membrane, *e. g.* that of the urethra.

(5) The occurrence of variations in the sterilising dose in repeated tests has been noted, and it appears that such differences are due to factors which cannot at present be completely defined.

(6) Methods of testing the antiseptic power of acriflavine by repeated

inoculation of a mixture of antiseptic and serum, and also by adding the antiseptic after the growth of organisms (*B. coli*) had occurred in the medium, have shown that rapid exhaustion or deterioration of the antiseptic in the medium does not occur. The latter experiment resembles more closely than the others the conditions met with in treating an infected wound. The striking fact has been elicited that a heavy growth of organisms is sterilised by the subsequent addition of the antiseptic with as great ease as are a few organisms added to the medium simultaneously with the antiseptic.

(7) Irregularity in the action of different concentrations of antiseptic in the same series has been observed, and the view has been advanced that, especially where the lethal effect is slowly produced, as with the compounds here considered, there is a critical concentration in the vicinity of which the balance may be inclined in favour of the organism or of the antiseptic by factors which at present escape complete analysis.

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TECHNIQUE.

ON PICRO-CARMINE.

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THE picro-carmine which Ranvier used for nearly all his staining work has been noted for its excellence for many years, but nobody else has been able to equal it, not even his pupils, many of whom have given formulæ for its preparation.

The reason may be that Ranvier worked with greater patience and slower methods than we do to-day, but chiefly, perhaps, because he always employed wet methods of staining and washing out under the cover-slip, where we now use paraffin or celloidin and mount in resin. It is also noteworthy that the only two workers that I have been able to find who recommend picro-carmine for staining, namely Stöhr and Mann, both advise the wet method and slow preparation after the manner of Ranvier.

In the course of time a number of different formulæ for picro-carmine have been given, most of which are mentioned in P. Mayer's excellent book on staining methods. The majority of them contain ammonia as a solvent for the carmine, but this makes their use impossible with paraffin sections, as the adhesive material used to fix them to the slide is dissolved and the section floats away.

From time to time I have tried many of these formulæ, as well as the liquid stains ready for use made by Grübler and Struer, but none of them were satisfactory.

If a solution of picric acid be gradually added to an alkaline solution of carmine a precipitate forms almost immediately, and the picric acid cannot, therefore, take an active part in the staining reaction. Moreover, the addition of magnesium picrate to magnesium-carmine, as mentioned by P. Mayer, does not give an adequate differential strain, because the picric acid, which functions as the differentiating medium, is wanting.

I therefore tried whether it was possible to combine both methods—the blending of a salt of picric acid with free picric acid—and after various

attempts, by altering the constituents and the mode of mixing, I discovered the following:

1. Magnesium-carmine (P. Mayer): 1 grm. carmine and 0·1 grm. magnesium oxide are boiled in 50 c.c. distilled water for 5 minutes. Cool and filter. Then 0·5 c.c. liquid phenol is added.

2. Magnesium picrate (P. Mayer): 50 c.c. ½ per cent. picric acid solution in distilled water boiled for some minutes with 0·25 grm. magnesium oxide. Cool and filter.

3. 1 per cent. picric acid in distilled water.

Mix 1 and 2. Then add 10 c.c. of 3, shaking constantly.

This gives an absolutely clear, deep red staining fluid, which on account of the phenol present remains unaltered for months, perhaps years.

The stain applied for 3–5 minutes to a celloidin or paraffin section produces a beautifully differentiated effect, and the nucleus especially is nicely stained, the best results being obtained after hardening in alcohol or sublimate. The sections do not become detached from the slide when they are fixed thereon by the method described by Mann, which is the one used at the Institute.

In virtue of the excess of free picric acid this stain has the further advantage that it can be used instead of picric acid for the beautiful method of staining bacteria devised by Claudius, which is much quicker and more certain than Gram's method for sections. In addition to this the tissue is simultaneously stained, so that the usual preliminary staining with lithium-carmine, differentiation with acid alcohol and washing with water can be dispensed with, thereby considerably shortening the procedure.

Combined with the Claudius method of staining a paraffin section the rationale is as follows:

(1) Xylol, 1 min.; (2) wash with xylol; (3) wash with alcohol; (4) methyl-violet (1 per cent. watery solution), 2–3 min.; (5) wash with water; (6) picro-carmine, 3–5 min.; (7) pour off the stain and dry with thick filter-paper; (8) decolorise with picric-aniline (aniline with a trace of picric acid added) till it becomes light red; (9) wash with xylol; (10) dry with filter-paper; (11) xylol-damar, cover-glass.

Gram-positive bacteria are stained blue, nuclei red, protoplasm orange, muscle and blood yellow, and connective tissue a rose tint.